PB81-171829

Determining Effect of Pollutants on the Immune System

Pennsylvania State Univ., University Park

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

Mar 81

U.S. Department of Commerce
National Technical Information Service
NTIS.

TECHNICAL REPORT DATA (Please read Instructions on the reverse before completing)		
1. REPORT NO. EPA-600/1-81-020	2.	3. RECIPIENT'S ACCESSION NO. PB81 171829
4. TITLE AND SUBTITLE Determining Effect of Pollutants on the Immune System		5. REPORT DATE March 1981 issuing date 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) A. Zarkower, J. Davis, F. Ferguson, and D. Strickler		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS The Pennsylvania State University University Park, PA 16802		10. PROGRAM ELEMENT NO. C9ZN1D 11. CONTRACT/GRANT NO. 68-02-2472
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED Final 14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES Project Officer: Judith A. Graham		

16. ABSTRACT

The purpose of this project was to determine the effects of fly-ash inhalation on the ability of animals to resist infections, neoplastic growth, and the development of hypersensitive responses. Mice were exposed to fly ash from two different sources, carbon black, and filtered ambient air only. Following exposures of various lengths (days to months), a variety of tests were done to determine the immunologic competence of lymphocytes, neutrophiles, and macrophages.

Fly ash had a suppressive effect on the ability of mice to respond to Escherichia coli antigens given by aerosol; this suppression was much less severe than that following exposures to carbon black. Fly ash had little effect on the ability of B and T lymphocytes to respond to mitogens and to be stimulated for cytolytic response against tumor cells. The cellular response of BCG-sensitized mice to purified protein derivative of tuberculin was enhanced.

The effect of fly ash on macrophages was more pronounced, involving decreased phagocytic activity, decreased antibody-dependent cytolosis, increased cytoxic activity against tumor cells, and decreased ability to activate T cell mitogenesis.

17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	94
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (9-73)

EPA-600/1-81-020
MARCH 1981

PB81-171826

DETERMINING EFFECT OF POLLUTANTS ON THE IMMUNE SYSTEM

by

A. Zarkower, J. Davis, F. Ferguson, and D. Strickler
The Pennsylvania State University
University Park, Pennsylvania 16802

68-02-2472

Project Officer

Judith A. Graham

Inhalation Toxicology Branch
Health Effects Research Laboratory
Research Triangle Park, North Carolina 27711

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NORTH CAROLINA 27711

DISCLAIMER

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, nonionizing radiation, environmental carcinogenesis, and the toxicology of pesticides, as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for nonionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The use of coal for power generation and for production of liquid and gaseous fuels is expected to increase in the near future. These processes can potentially release into the environment a number of substances capable of reacting with biological systems. As fly ash will be a significant coal-derived pollutant, it is important to determine its potential hazard to human health.

F. G. Heuter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

This research project was initiated in order to determine the consequences of fly-ash inhalation on the animals' ability to resist infections, neoplastic growth and the development of hypersensitive responses.

Mice were exposed to fly-ash obtained from two sources, carbon black and filtered ambient air only. Following exposures lasting various intervals of time (days to months) a variety of tests were done to determine the immunologic competence of lymphocytes, neutrophiles and macrophages.

Fly-ash had a suppressive effect on the ability of mice to respond to E.coli antigens given by aerosol. This suppression was much less severe than that obtained after exposures to carbon black. Fly-ash had little, if any, effect on the ability B and T lymphocytes to respond to mitogens and to be stimulated for cytolytic response against tumor cells. The cellular response of BCG sensitized mice to PPD was enhanced.

The effect of fly ash on macrophages was more pronounced; a decreased phagocytic activity; a decrease in antibody dependent cytolysis; an increase in cytotoxic activity against tumor cells; a decreased ability to activate T cell mitogenesis.

This report was submitted in fulfillment to Contract No. 68-02-2472 by the Center for Air Environment Studies of Pennsylvania State University under the sponsorship of U.S. Environmental Protection Agency. This report covers the period 9/29/76 to 7/31/80.

CONTENTS

Foreword iii
Abstract iv
Figures vi
Tables ix

Introduction 1

Experimental Procedures 4

Results 13
Part I 13
Part II 30
Part III 47
Part IV 63
Part V 66
Part VI 73

Summary 82

Conclusions 82

Appendix 83

FIGURES

Number		Page
1	Effects of fly ash and carbon inhalations: Number of antibody-forming cells in spleens in response to E. coli antigen given as an aerosol.	15
2	Effects of fly ash and carbon inhalations: Number of antibody-forming cells/10^6 spleen cells in response to E. coli antigen given as an aerosol.	16
3	Effects of fly ash and carbon inhalations: Number of antibody-forming cells/10^6 mediastinal lymph node cells in response to E. coli antigen given as an aerosol.	17
4	Effects of fly ash and carbon inhalations: Serum agglutinating activity in response to E. coli antigen given as an aerosol.	18
5	Effects of fly ash and carbon inhalations: ^3H - thymidine incorporation into Con A responsive spleen cells	19
6	Effects of fly ash and carbon inhalations: ^3H - thymidine incorporation into LPS responsive spleen cells	20
7	Effects of fly ash and carbon inhalation: ^3H - thymidine incorporation into a one-way mixed lymphocyte culture.	21
8	Effects of fly ash and carbon inhalations: cytolytic activity of stimulated T lymphocytes from spleens.	22
9	Effects of fly ash and carbon inhalations: Number of antibody-forming cells in spleens in response to E. coli antigen given as an aerosol.	39
10	Effects of fly ash and carbon inhalations: Number of antibody-forming cells/10^6 spleen cells in response to E. coli antigen as an aerosol.	40
11	Effects of fly ash and carbon inhalations: Number of antibody-forming cells/10^6 mediastinal lymph node cells in response to E. coli antigen given as an aerosol.	41

FIGURES (Continued)

Number		Page
12	Effects of fly ash and carbon inhalations: Serum agglutinating activity in response to *E. coli* antigen given as an aerosol. . .	42
13	Effects of fly ash and carbon inhalation: ^{3}H - thymidine incorporation into ConA responsive spleen cells.	43
14	Effects of fly ash and carbon inhalation: ^{3}H - thymidine incorporation into LPS responsive spleen cells	44
15	Effects of fly ash and carbon inhalation: Cytolytic activity of stimulated T lymphocytes from spleens.	45
16	Effects of fly ash and carbon inhalation: *In - vitro* phagocytosis of *E. coli* bacteria by pulmonary macrophages	46
17	Antibody dependent cell-mediated cytotoxicity of mouse lung cells.	51
18	Antibody dependent cell-mediated cytotoxicity of guinea pig lung cells at various effector to target cell ratios.	52
19	Antibody dependent cell-mediated cytotoxicity of hamster lung cells at various effector to target cell ratios.	53
20	Effects of intratracheal silica injections on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages (1:1 effector/target cell ratio)	54
21	Effects of intratracheal silica injections on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages . .	55
22	Effects of intratracheal fly ash injections on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages . .	56
23	Effects of silica inhalation on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages.	57
24	Effects of fly ash inhalation on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages.	58
25	Effects of intratracheal particulate injections on macrophage mediated cytotoxicity.	59
26	Effects of fly ash inhalation on T lymphocyte mitogenesis with PHA.	60
27	Effects of fly ash inhalation on T lymphocyte mitogenesis with PHA.	61

FIGURES (Continued)

Number		Page
28	Effects of fly ash inhalation on macrophage mediated cytotoxicity by alveolar macrophages.	62
A	Section of lung through bronchiole(br) showing accumulation of PAMA (small arrow) along epithelium. Also shown is an accumulation of lymphoid cells (parabronchial lymphoid infiltration) to bronchiolar epithelium (large arrow). x100, #80-35.	76
B	Section through mediastinal lymph node. Note accumulation of dark pigment in peripheral portion of lymph node (arrow). x100, #80-35	77
C	A higher magnification of figure A, showing the relationship of PAMA (arrow), the lining epithelium, and a normal alveolus (alv). x400	78
D	Same section as figure C, above, but examined under polarized light. Note birefringent particles in periphery of PAMA (arrows). x400.	79
E	Section of lung showing deposition of PAMA (arrow) in Alveoli near terminal bronchiole(br). x100	80
F	A higher magnification of figure E, above, observed under polarized light. Note presence of birefringent particles in PAMA (arrows). x400	81

TABLES

Number		Page
1	Particulate Exposures Concentrations Expressed as $\mu g/m^3$ of Air. Series I Experiments	23
2	Response to Stimulation With Aerosols of E.coli 055:B5 Antigen. Series I Experiments	24
3	Antibody Response of Fly Ash-Exposed Mice to LPS Aerosol	25
4	Antibody Response of Fly Ash-Exposed Mice to Intratracheal Injection of LPS	25
5	Lymphocyte Transformations - Series I. Disintegrations Per Minute $\pm$ S.E.	26
6	Lymphocyte Transformations. Series I Experiments.	27
7	Body and Spleen Weights of Mice Used for Lymphocyte Transformations $\pm$ S.E. Series I Experiments	28
8	Cytolytic Activity of Spleen Cells Expressed as Log_{10} Number of Spleen Cells Needed for 10% Lysis $\pm$ S.E. Series I Experiments	29
9	Number of Antibody Forming Cells in Pooled Mediastinal Lymph Nodes/10^6 Lymph Node Cells Response to Stimulation With E.coli Antigen. Series I Experiments	29
10	Concentration of Particulates During the Series II Experiments Expressed as $\mu g/m^3$ of Air.	31
11	Response of Mice to Stimulations With Aerosol of E.coli 055:B5 Antigen: Series II Experiments.	32
12	Lymphocyte Transformation - Series II (Spleen Cells) Disintegrations Per Minute $\pm$ S.E.	33
13	Lymphocyte Transformations - Series II (Spleen Cells) Stimulation Index $\pm$ S.E.	34
14	Number of Antibody Forming Cells in Pooled Mediastinal Lymph Nodes/10^6 Lymph Node Cells	35

TABLES (Continued)

Number		Page
15	Spleen Weights in Grams ± S.E. Series II Experiments.	36
16	Cytolytic Activity of Spleen Cells Expressed as Log_{10} Number of Spleen Cells Needed for 10% Lysis ± S.E. Series II Experiments.	37
17	*In Vitro* Phagocytosis of *E. coli* Bacteria by Alveolar Macrophages: Per Cent of Macrophages Containing *E. coli* ± S.E.	38
18	. . . . .	50
19	. . . . .	63
20	. . . . .	64
21	. . . . .	65
22	. . . . .	68
23	. . . . .	68
24	. . . . .	69
25	. . . . .	71

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD BEGIN SECTIONS HERE

INTRODUCTION

The lung is the principal portal of entry for airborne substances and since the surface of the respiratory tract exposes an extremely large area to the environment it is the primary exposure site for gases and for particles smaller than 2μ in diameter. After inhalation, toxic substances may interact with surface proteins and cells, or may enter the body and be carried to other organs, such as: lymphoid tissues, liver and kidneys. Though other organs may well be involved, the ones named are the principal ones in clearance, detoxification, and excretion. Other portals of entry into the body also exist for airborne substances. These are the gastrointestinal tract and the skin. Substances entering the mouth both directly or as a result of being coughed up from the respiratory tract are swallowed. Absorption through the skin is usually quite minor, but in some cases it may have profound effects on physiological functions and may stimulate hypersensitive responses. Thus, exposure to airborne substances can result in absorption by several routes with the lung accepting the greatest burden.

Inhalation of toxic substances can cause disease two ways. One way is by direct damage to lung tissues. This type of damage often follows exposure to high concentrations of toxic minerals (silica, asbestos) or gases (ozone, nitrogen dioxide). The second way inhaled substances can cause damage is indirectly by affecting other organ systems. Examples of substances which can cause indirect effects are: lead which can affect a variety of tissues (gastrointestinal tract, nervous system and blood); cadmium which can cause kidney damage and osteomalacia; and, mercury which can affect the central nervous system.[1,2] Many substances including gases and organic and inorganic particulates have been found to affect immunological responses in the body.[3-11] A variety of effects have been observed ranging from suppression to enhancement of antibody formation, suppression or enhancement of cellular immunoresponses and phagocytic activities of macrophages. Changes in immunocompetence have many implications. Decreased antibody responses may lead to increased susceptibility to infectious diseases. Increased antibody stimulation of specific types may lead to increased resistance to infectious diseases, but may also lead to immunopathological conditions such as immediate hypersensitivity and immune complex diseases. Decreased cellular immune responses may lead to decreased resistance to certain infectious agents and certain neoplastic processes, while increased cellular immunocompetence may manifest itself as delayed type hypersensitivity. Changes in neutrophile and macrophage functions also can affect resistance to both infectious and neoplastic diseases. Thus changes in immunological capabilities can eventually affect health and longevity.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-287 (Cin.) (4-76)

The near future will witness the proliferation of a variety of alternate energy sources and their attendant pollutants. In particular, expanded use of coal for power generation and as stock for liquid and gaseous fuels is expected to yield a number of products (SO_2, NO_2, hydrocarbons, particulates, etc.) which could react with biological systems. Fly-ash will be a large component of the coal-derived air pollutants and it is composed of many elements capable of causing cell damage. Since inhaled particulates will come into intimate contact with cells of the immune system (macrophages, lymphocytes) the possibility exists that changes in immunological responses may occur leading ultimately to decreased ability to cope with infectious agents and neoplastic cells.

This project was designed to test the effects of fly-ash inhalation on cells involved in the immune response and their functions. The animals were exposed to fly ash, and in some cases carbon, in especially designed chambers. After variable amounts of time the exposed and control animals were removed from the chambers and determinations were done for immunological competence and histological changes in cells and organs.

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE

REFERENCES

1. Goldsmith, J. R., and T. Friberg. Effects of Air Pollution on Human Health. In: Air Pollution, Vol. II, 3rd Ed., A. C. Stern, ed. Academic Press, New York, 1977. pp. 457-610.

2. Coffin, D. L., and H. E. Stokinger. Biological Effects of Air Pollutants. In: Air Pollution, Vol. II, 3rd Ed., A. C. Stern, ed. Academic Press, New York, 1977. pp. 231-360.

3. Koller, L. D., J. Roan, and N. I. Kerkvliet. Mitogen Stimulation of Lymphocytes in CBA Mice Exposed to Lead and Cadmium. Environmental Research, 19:177-188, 1979.

4. Holt, P. G., L. M. Finlay-Jones, D. Keast, and J. M. Papamitron. Immunological Function of Mice Chronically Exposed to Nitrogen Oxides. (NO_x). Environmental Research, 19:154-162, 1979.

5. Graham, J. A., F. J. Miller, M. J. Daniels, E. A. Payne, and D. E. Gardner. Influence of Cd, Ni, and Cr on Primary Immunity in Mice. (Personal Communication).

6. Osebold, J. W., S. L. Owens, Y. C. Zee, W. M. Dotson, and D. D. LaBarre. Immunological Alterations in the Lungs of Mice Following Ozone Exposure: Changes in Immunoglobulin Levels and Antibody-Containing Cells. Arch. Environ. Health, pp. 258-264, July-August 1979.

7. Fenters, J. D., J. M. Bradof, C. Aranyi, K. Ketels, R. Ehrlech, and D. E. Gardner. Health Effects of Long-Term Inhalation of Sulfuric Acid Mist - Carbon Particle Mixtures. Environmental Research, 19:244-257, 1979.

8. Zarkower, A., and W. Morges. Alteration in Antibody Response Induced by Carbon Inhalation: A Model System. Infection and Immunity, 5:915-920, 1972.

9. Zarkower, A. Alterations in Antibody Response Induced by Chronic Inhalation of SO_2 and Carbon. Arch. Environ. Health, 25:45-50, 1972.

10. Miller, S. D., and A. Zarkower. The Effects of Carbon Dust Inhalation on the Cell Mediated Immune Response in Mice. Infection and Immunity, 9(3):534-539, 1974.

11. Miller, S. D., A. Zarkower. Alterations of Murine Immunologic Responses After Silica Dust Inhalation. J. Immunol., 113(5):1533-1543, 1974.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-287 (Cin.)
(4-76)

EXPERIMENTAL PROCEDURES

EXPOSURE FACILITIES

Figure 1 is a plumbing schematic of the dust exposure system with Figure 2 showing the details of the dust generation and sampling devices. Air was admitted into the system in two ways. First, clean dry compressed air was used as a carrier gas for the generated aerosol; the flowrate being one cfm. In order to be certain that all animals received air of the same relative humidity, the control chambers received one cfm of the same air. The air pressure was regulated and fed through identical orifices to the inlet pipe of the chambers. The second source of air was through absolute filters attached to the inlet of each chamber and was drawn in by means of a vacuum pump. The two air streams were mixed before entering the chamber and by using a butterfly valve the pressure inside the chamber was maintained at a few tenths of an inch of water below atmospheric pressure.

The exposure chambers were equipped with dry dust generators originally described by Crider[4]. Compressed air at preset intervals was pulsed into a dust bed and the particles were forced and drawn (by venturi action) into an air stream passing through the top of the generator cavity.

Intermittent sampling was done to determine both total mass, and size distribution of the particles.

Immunological Studies

Animals--Female BALB/c mice were matched by age and weight for each experiment. They received pelleted food and water, both supplied ad libitum.

Antigenic Stimulations--Aerosol exposures were done in a Model A-4 Airborne Infection Apparatus obtained from Tri-R Instruments Inc., Rockville Centre, New York. Ten milliliters of antigen suspension were used in each run. Exposures usually lasted 60 minutes at a setting of 120 on the compressed air flowmeter (5.3 l/min) and 190 on the main air flowmeter (47.3 l/min.). *E. coli* antigen concentration was 50 mg/ml, producing an aerosol containing approximately 90 $\mu g/ft^3$ (4). *E. coli* lipopolysaccaride antigen was used at 1 mg/ml while washed sheep red blood cell (SRBC) suspensions were used at concentrations ranging from 10 to 40 percent.

Intravenous immunizations were given in a volume of 0.25 ml in the lateral tail vein. Antigen type and concentration varied, depending on the experiment.

Intratracheal Instillations--Deposition of material into the trachea was used both with antigens as a means of pulmonary immunization and with particulate materials for certain microscopy studies performed on mice sedated with pentobarbital sodium. A 0.05 ml. aliquot of antigen or particulate was introduced into the trachea with a 27 g needle on a 1 ml. tuberculin syringe. Concentrations used varied in different experiments.

In-Vitro Lymphocyte Transformation--The lymphocyte transformation technique was modified from that of Adler et al. (1-2). Lymphoid organs were excised asceptically and single cell suspensions were prepared. The final cell suspensions were adjusted to 2×10^7 cells. Mitogens were prepared in complete RPMI (with 5% human serum, 100 IU penicillin and 100 µg of streptomycin per ml.) at the following concentrations: Con A at 15 µg/ml, PHA-P at 2.5 µl/ml, and LPS at 15 µg/ml. Triplicate cultures were set up in the microtiter plate wells by adding 0.05 ml of the cell suspensions along with 0.1 ml of mitogen solution (or complete RPMI in the case of control cultures). Culture plates were placed in humid 5% CO_2 incubator at 37°C. Con A and PHA cultures were incubated for 48 hours and LPS for 72 hours. 24 hours before harvest, 0.5 µCi of ^3H thymidine was added to the cultures in a volume of 0.05 ml of incomplete RPMI medium and the cultures were returned to the incubator.

Following incubation the cultures were washed onto glass fiber filters using saline. The glass fiber discs containing the ^3H-labelled cells were removed from the filter strips and placed into scintillation vials. Any water remaining in the discs was removed by air drying the open vials for 48 hours or by placing the vials in a 70-80°C drying oven for several hours. When the filter discs were dry and cooled to room temperature, 0.1 ml of NCS was added to each disc. After allowing about 5 min. for the NCS to solubilize the material on the discs, 10 ml of scintillation fluid was added to each vial and the vials were mixed and counted. Counts per minute were calculated for each culture and triplicate cultures were averaged. The average values of mitogen-stimulated cultures were divided by their respective control values to give the transformation ratio.

Mixed Lymphocyte Culture (3)--A spleen was removed asceptically from a $C_{57}BL/6$ mouse and a single cell suspension was prepared in the same manner as the BALB/c spleens in the lymphocyte transformation (several spleens may be pooled if needed). The cell suspension in RPMI-1640 wo serum was adjusted to 3×10^7 cells/ml and 0.1 ml of a 2 mg/ml solution of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added per ml. of suspension. The mixture was incubated at 37°C for 30 min. and washed 3X in RPMI 1640 wo serum, then resuspended in RPMI 1640 w 5% serum, 1% Pen/Strep and adjusted to 10^7 cells/ml. 0.1 ml of this suspension was added to each microculture well, just as the mitogen solutions were added in the lymphocyte transformation. Cultures were ^3H labeled after 48 hrs., and harvested after 72 hrs.

Transformation of Spleen Cells Following Subcutaneous Sensitization with M. tuberculosis--Subcutaneous sensitizations were accomplished by injecting the mice in four sites in the dorsal thoracic area with 0.5 ml. of complete Freund's adjuvant (Baltimore Biological Laboratory, Baltimore, Maryland)

supplemented with 1.0 mg/ml of M. bovis BCG. The transformation of spleen cells was measured by the technique described under "In-Vitro Lymphocyte Transformation", with stimulation measured in response to purified protein derivative of tuberculin (PPD).

Delayed Type Hypersensitivity (DTH) in the Murine Lung--This assay was used to measure the accumulation of radiolabelled lymphocytes at the site of antigen deposition of a previously sensitized animal.

Mice were sensitized by subcutaneous injection of BCG in complete adjuvant. Twenty-one to 28 days later the mice were injected intravenously with radiolabelled mononuclear cells obtained from unsensitized syngeneic mice. A few hours later the sensitized mice were challenged with an intratracheal injection of PPD. 48 hours later the lungs were removed and the accumulation of radioactive cells in the lungs was determined by measuring the amount of radioactivity present in the lungs as a ratio of the total radioactivity injected.

Localized Hemolysis in Gel--The plaque assay was modified after the method of Dresser and Wortis (5). To measure the number of anti-E. coli LPS producing cells, we use a modified procedure. The SRBC were diluted to 10% with 1 mg/ml of previously boiled LPS (1 hr.) and were incubated for 1 hr. at 37°C. The cells were then washed three times with buffered saline and diluted to a 10% suspension. Agarose at 1.2% in Hanks' balanced salt solution (HBSS) was used as the base layer. The overlay suspension of indicator and lymphoid cells in 0.6% agarose was incubated for 1 hour at 37°C., followed by a second incubation with guinea pig complement for 1 hour. For estimation of the secondary response (IgG producing cells) the overlay was incubated with a previously titered 0.1 ml of rabbit antimouse serum. The number of plaques in plates without antisera (IgM plaques) are subtracted to give an estimate of IgG producing cells.

Total organ cell counts were done on a Coulter Counter (Model Zf). The plaques were counted under a stereoscopic microscope using a Sensaur Bacti Counter.

Serology--Serum antibody titers were expressed as the reciprocal of the highest dilution of serum that produced complete agglutination of the LPS-coated SRBC. Microtiter plates and microdiluters were used for the assays. The values obtained were converted to $\log_{10}$ for statistical analysis.

Cell Mediated Cytolysis--The cell-mediated cytotoxicity assay was essentially that described by Brunner, et al. (8) and Henney (9). A mouse EL-4 lymphoma of C57Bl/6 ($H\text{-}2^b$) strain was maintained in the ascites form by weekly passage of 10^7 cells in C57Bl/6 mice. Target cells (lymphoma cells) for the in-vitro cytolysis were prepared as described by Henney (9). The ascites fluid was aspirated into a syringe containing Tris-saline buffer, pH 7.4 and the cells were pelleted at 400 X g for five minutes and washed once in the buffer. Then 10^7 cells were suspended in 0.5 ml of Eagle's MEM containing 100 units of penicillin and 100 ug of streptomycin per ml and incubated for 30 minutes at 37°C with 50 uCi of sodium ^{51}chromate (New England

Nuclear, S.A. of 6.7 Ci/m mol). The cells were then washed three times in 10.0 ml volumes of MEM and adjusted to 10^6 cells/ml in MEM containing 10% FCS (complete MEM).

Single cells suspensions were prepared from spleens of Balb/c mice ($H\text{-}2^d$) sensitized i.p. 10-11 days prior to the assay with 3×10^7 EL-4 cells ($H\text{-}2^b$), using the same method as in the immuno-plaque assay. Erythrocytes were lysed by a single wash in 0.83% ACT at 37°C. Cell debris was removed by centrifuging for 20 seconds at 165 x g, discarding the pellet and recentrifuging the supernatant at 400 x g for five minutes. This pellet is then washed twice in complete MEM and the cell concentration was adjusted to 10^7 viable cells per ml.

Labeled target cells, 10^5 in 0.1 ml, were added to varying numbers of sensitized spleen lymphocytes (5×10^5 to 5×10^6) and the total volume adjusted to 1.0 ml with complete MEM in 10 x 75 mm plastic test tubes. This produced lymphocyte/target cell ratios of 5:1 to 50:1. The tubes were then incubated at 37°C for four hours in a humidified, 5% CO_2 atmosphere without agitation. Following the culture period, the tubes were centrifuged at 1000 x g for five minutes and a 0.5 ml aliquot of the supernatant was counted for released ^{51}Cr on a gamma-ray spectrometer (Packard Instruments Model 3375 Auto Gamma Spectrometer, Downers Grove, Illinois).

Control cultures consisted of spleen cell suspensions from unsensitized mice and cultures to which no sensitized lymphocytes were added. These were usually found to be the same and this spontaneous release of ^{51}Cr did not exceed 5-10% of the total ^{51}Cr content (8-9). The total ^{51}Cr content was determined by counting a 0.5 ml aliquot of vortexed target cells which are incubated for the entire culture period. The percentage of specific cytolysis was calculated with the following formula:

$$\% \text{ Specific Release} = \frac{\begin{array}{l}{}^{51}Cr \text{ cpm in the presence of immune lymphoid cells} \\ - \\ {}^{51}Cr \text{ cpm in the presence of normal lymphoid cells}\end{array}}{\begin{array}{l}\text{Total } {}^{51}Cr \text{ cpm} \\ - \\ {}^{51}Cr \text{ cpm in the presence of normal lymphoid cells}\end{array}}$$

Alveolar Macrophage Phagocytic Index

1. Phagocytosis of Escherichea coli

Lungs from mice were excised asceptically and single cells suspensions were prepared by teasing through 60-mesh stainless steel wire screens. These cells were suspended in 2.0 ml of minimal essential medium (MEM-Grand Island Biological Co.) supplemented with 20% fetal calf serum, 50 units penicillin, and 50 µg streptomycin per milliliter (complete MEM). The suspension was added to a Leighton cover glass culture tube and incubated for 24 hr. at 37°C in a humidified 5% CO_2 atmosphere. After the initial incubation period, the growth medium was aspirated off and the coverslips were washed three times with MEM. Finally, 2.0 ml of complete MEM were added to the culture tubes

along with 0.1 ml of an overnight broth culture of E. coli 055:B5. grown in tryptic soy broth and washed two times with Hanks' balanced salt solution (HBSS). The cultures were reincubated for 2 hr. and washed two times with HBSS. The coverslips were then fixed for 10 min. in a 5% formalin:95% HBSS solution and dehydrated and stained according to the method of Patton (6). Two hundred cells were counted and the percentage of cells phagocytizing the bacteria were determined.

2. Phagocytosis of Staphylococcus aureus

Alveolar macrophages were obtained by lavage using phosphate buffered saline. After washing, cells were suspended in complete MEM, placed in Leighton tubes and incubated for one hour. Following incubation, $2.5X10^7$ Staphylococcus aureus cells were added to the culture tube followed by 0.5 hour incubation period. The media containing unphagocytized bacteria was aspirated off and lysostaphin was added. Macrophages were then stained and using a light microscope their ability to phagocytize S. aureus was determined. The results were tabulated as a percentage of macrophages phagocytizing a certain number of bacteria.

Macrophage-mediated Cytotoxicity Assay

Alveolar macrophages were lavaged with HBSS from the lungs of hamsters that had been sensitized i.t. with 4 mg BCG and challenged with BCG 5 days prior to the assay. Cells were spun down, resuspended in complete McCoy's media, and the cell suspension at a concentration of 5×10^5 cells/ml was added in duplicate to wells of a Linbro plate and allowed to adhere at 37°C in a humidified atmosphere. Two hours later, the alveolar macrophage monolayers were washed with warm HBSS to remove the nonadherent cells.

Hamster H50 tumor target cells were prelabeled with 50 μCi of tritiated thymidine. The tumor target cells were released from the monolayers after 24 hr. of radioactive incorporation, washed twice and resuspended in complete McCoy's media. Target cells were then added at a concentration of 5×10^3 cells per well to attain a 100:1 effector to target ratio. The cultures incubated for 5 hours at 37°C and then were washed three times to remove the nonadherent target cells. Complete media was added and the plates were then incubated for 120 hr. at 37°C.

After the 5 day incubation period, the culture medium was removed and centrifuged. A 0.5 ml aliquot was placed in 10 ml Aquasol and the radioactivity released by the tumor targets was determined in a liquid scintillation counter. Target cells were lysed with 0.5% sodium lauryl sulfate to determine the total radioactivity incorporated by the tumor target cells. The percent target cell lysis was calculated by:

$$\frac{\text{cpm with macrophages} - \text{cpm without macrophages}}{\text{total cpm}} \times 100.$$

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

Antibody-dependent Cell-mediated Cytotoxicity by Macrophages

Lung cells were obtained from hamsters by lavage with warm HBSS or CRPMI and 12 mM lidocaine. Cells were spun down, washed twice in HBSS, resuspended in CRPMI, and adjusted to a concentration of 1×10^6/ml. A volume of 350 µl (3.5×10^5 cells) was then added in duplicate to the wells of a flat-bottomed microtiter plate and incubated at 37°C for 1.5 hr. The macrophage monolayers were then washed three times with warm HBSS to remove nonadherent cells. Ten µl of a 1:160 dilution of rabbit anti-CRBC sera was added to each well along with 3.5×10^4 ^{51}Cr-labeled CRBC targets in a volume of 20 µl. Antisera and CRBC targets were then incubated for 30 min. at 4°C. CRPMI was added for a final volume in each well of 0.2 ml and the plates were incubated at 37°C, 5% CO_2 in air atmosphere.

At the end of the 4 hr. incubation period, the plates were centrifuged and half the supernatant was removed and counted in a gamma counter. Spontaneous release was determined by incubating only labeled CRBC and anti-CRBC sera. Maximum lysis was obtained by incubating 3.5×10^4 CRBC in 0.5% sodium lauryl sulfate for the duration of the assay. Percentage of chromium release was calculated as:

$$\% \text{ release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

T cell Mitogenesis by Macrophages

Lung macrophages were obtained from hamsters by lavage with warm HBSS or CRPMI and 12 mM lidocaine. The cells were spun down and washed in lidocaine-free medium twice. Single cell suspensions of alveolar macrophages were aliquoted into flat-bottomed microtiter plates at different concentrations in duplicate. Macrophages were added to give a final concentration of 20, 10, 1 and 0.1% alveolar macrophages in the lymphocyte cultures. The suspensions were incubated for 2 hr. and the nonadherent cells were washed off three times with warm HBSS.

A "T-enriched" population of spleen cells was then added to the macrophage monolayer at a concentration of 2.0×10^5 cells per well in a volume of 100 µl. The mitogen PHA was subsequently added in 50 µl volumes, at a concentration of 1.67 µl/well. The cultures were then incubated for 24 hr. at 37°C. The cultures were pulsed with 0.5 uCi tritiated thymidine and incubated 24 hr. further. The plates were then harvested and the amount of ^3H-DNA synthesis was determined by liquid scintillation counting. Data were expressed as the log counts per minute per culture.

Examinations of Tissues Using Scanning Electron Microscopy--A technique for the examination of alveolar macrophages from fly-ash exposed and control mice was worked out. Cells washed-out from lungs were allowed to adhere to glass and prepared for microscopy by conventional methods. Those to be examined using x-ray analysis were carbon-coated and those to be examined for membrane changes were carbon and gold-coated.

BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
BEGIN LAST LINE OF TEXT
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD, BEGIN SECTIONS HERE

Energy Dispersive X-Ray Analysis

The electron microprobe used was an EDAX unit attached to a Phillips 300 electron microscope. The unit also contained a scanning component and could be operated in a scanning transmission mode. It was also capable of x-ray mapping. The system identified and measured the x-ray energy of the elements whose atomic number was 11 or greater. Detection of 10^{-18} grams of an element was the sensitivity obtained. By using the x-ray mapping mode of operation the intra- and extracellular location of an element of interest was determined. More detailed analysis using the transmission or scanning transmission mode was then made.

The data generated by the equipment was used to obtain both qualitative and quantitative information of the particular elements under study.

Statistical Methods

Analyses of variance were performed and means for each group were compared according to Newman-Keul's sequential range test as described by Steel and Torrie (7).

1/2"
9-1/8"
BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

REFERENCES

1. Adler, W. H., T. Takiguchi, B. Marsh, and R. T. Smith. Cellular Recognition by Mouse Lymphocytes In Vitro. I Definition of a New Technique and Results of Stimulation by Phytohemagglutinin and Specific Antigens. J. Exp. Med., 131:1049-1078, 1970.

2. Adler, W. H., T. Takiguchi, and R. T. Smith. Preparation and Assay of Mouse Mutogenic Factors. In: In Vitro Methods in Cell-Mediated Immunity, R. B. Bloom and P. R. Glade, eds. Academic Press, New York and London, 1971. pp. 433-439.

3. Adler, W. H., T. Takiguchi, B. Marsh, and R. T. Smith. Cellular Recognition by Mouse Lymphocytes In Vitro II. Specific Stimulation by Histocompatibility Antigens in Mixed Cell Culture. J. Immunol., 105:984-1000, 1970.

4. Zarkower, A., and W. Morges. Alterations in Antibody Response Induced by Carbon: A Model System. Infec. and Immun., 5:915-920, 1972.

5. Zarkower, A. Alterations in Antibody Response Induced by Chronic Inhalation of SO_2 and Carbon. Arch. Environ. Health, 25:45-50, 1972.

6. Miller, S. D., and A. Zarkower. Alterations of Murine Immunologic Responses After Silica Dust Inhalation. J. Immunol., 113:1533-1543, 1974.

7. Steel, R. G., and Torrie. Principles and Procedures of Statistics. McGraw-Hill Book Co., New York, 1960.

8. Brunner, K. T., J. Manuel, J. C. Cerottini, and B. Chapus. Quantitative Assay of the Lytic Action of Immune Lymphoid Cells on ^{51}Cr-Labelled Allogeneic Target Cells In Vitro: Inhibition by Isoantibody and by Drugs. Immunol., 14:181-196, 1968.

9. Henney, C. S. Quantitation of the Cell-Mediated Immune Response I. J. Immunol., 107:1558-1566, 1971.

10. Berger, A. E., and D. B. Amos. A Comparison of Antibody-Dependent Cellular Cytotoxicity Mediated by Murine and Human Lymphoid Cell Populations. Cell. Immunol., 33:277-290.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-287 (Cin.)
(4-76)

11. Zighelboim, J., B. Bonavida, and J. L. Fahey. Heterogenous Populations of Cytotoxic Cells in the Peritoneal Cavity of Balb/c Mice Immunized with Allogeneic E14 Leukemia Cells. Cell. Immunol., 12:280-289, 1974.

12. Norbury, K. C., and I. J. Fidler. In Vitro Tumor Cell Destruction of Syngeneic Mouse Macrophages:Methods for Assaying Cytotoxicity. J. Immunol. Methods, 7:109-122, 1975.

13. Zwilling, B. D., and L. B. Campolito. Destruction of Tumor Cells by BCG-Activated Alveolar Macrophages. J. Immunol., 119:838-841, 1977.

14. Hunninghake, S. O., and A. S. Fauci. Immunological Reactivity of the Lung. II Cytotoxic Effector Function of Pulmonary Mononuclear Cells Subpopulations. Cellular Immun., 26:98-104, 1976.

15. Holt, P. G. Alveolar Macrophages. II. Inhibition of Lymphocyte Proliferation by Purified Macrophages from Rat Lungs. Immun., 37:429-436, 1979.

RESULTS

The studies on the effects of fly-ash and carbon inhalation on immunological response and tissue morphology were divided into several parts.

PART I

Mice in groups of eight were placed in each of the three exposure chambers; control (filtered air only), fly ash (obtained from Penn State power plant), and carbon (all-gas channel black obtained from Sid Richardson Co., Fort Worth, Texas). The total concentrations of these generated particulates and the proportion of generated particulates that were smaller than 2.1 μ (respirable range) are shown in Table 1. This table also indicates the exposure periods of the completed experiments.

The effects of these exposures are shown in Figures 1, 2, 3, 4, 5, 6, 7, and 8. The effects of the exposures on the numbers of antibody-forming cells in the spleens subsequent to stimulations of the mice with E. coli aerosols are shown in Figures 1 and 2. Both fly ash and carbon decreased these responses with the carbon effects being much more pronounced. The statistical tests were done after $\log_{10}$ transformations of the data. This treatment of data is more rigorous and decreased the statistical significance of fly ash after 21 and 35 days of exposure. The effects of the exposures on antibody-forming activity in the mediastinal lymph nodes is shown in Figure 3. Enhancements after 7 days of exposure were followed by suppression after 21 days and a recovery after 35 and 56 days of exposures. The values in Figure 3 are per 10^6 cells. Carbon and fly ash both caused a hypertrophy of the lymph nodes with an increase in the number of lymphocytes at all times after exposure. The agglutinating activity in the sera was found to follow closely the pattern of suppression seen in the spleens (Figure 4).

Following a 9 month exposure to fly ash at somewhat higher concentrations than the preceding experiments a highly significant decrease in splenic antibody forming cell response was measured in mice exposed to LPS either by aerosol (Table 3) or intratracheal injection (Table 4). Although plaque forming cell responses in the MLN were lower in both cases, these changes were not statistically significant ($p < 0.05$).

Fly ash and carbon inhalations caused few changes in the lymphocyte responses to T and B cell mitogens. An absolute decrease to Con A was found in lymphocytes from fly-ash exposed mice after 21 days (Figure 5). The response of the B cell population to LPS did not appear to be significantly affected (Figure 6).

Other functions of T cells showed little effect due to the exposures. The recognition activity of the cells from fly-ash exposed mice was decreased after 21 days of exposure (Figure 7). No significant change was found in the cytolytic capacity of T cells after any period of exposure (Figure 8).

Numerical expression of the data described in the various figures is shown in Tables 2 to 9.

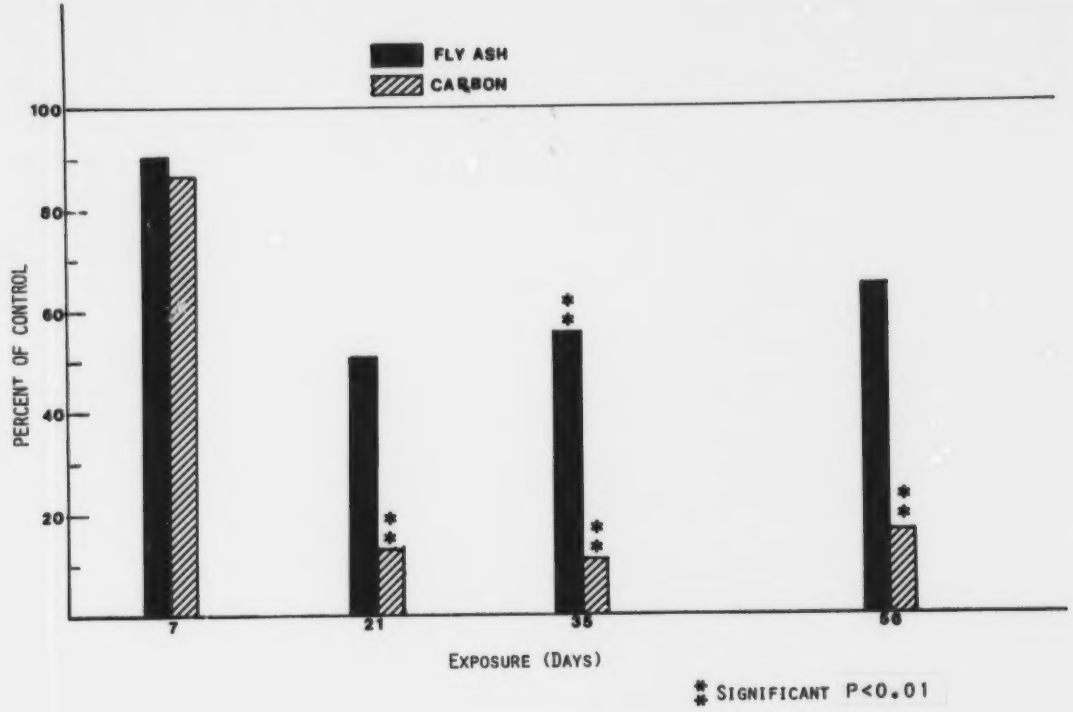


Figure 1. Effects of fly ash and carbon inhalations: Number† of antibody-forming cells in spleens in response to E. coli antigen given as an aerosol.

† values expressed as geometric means

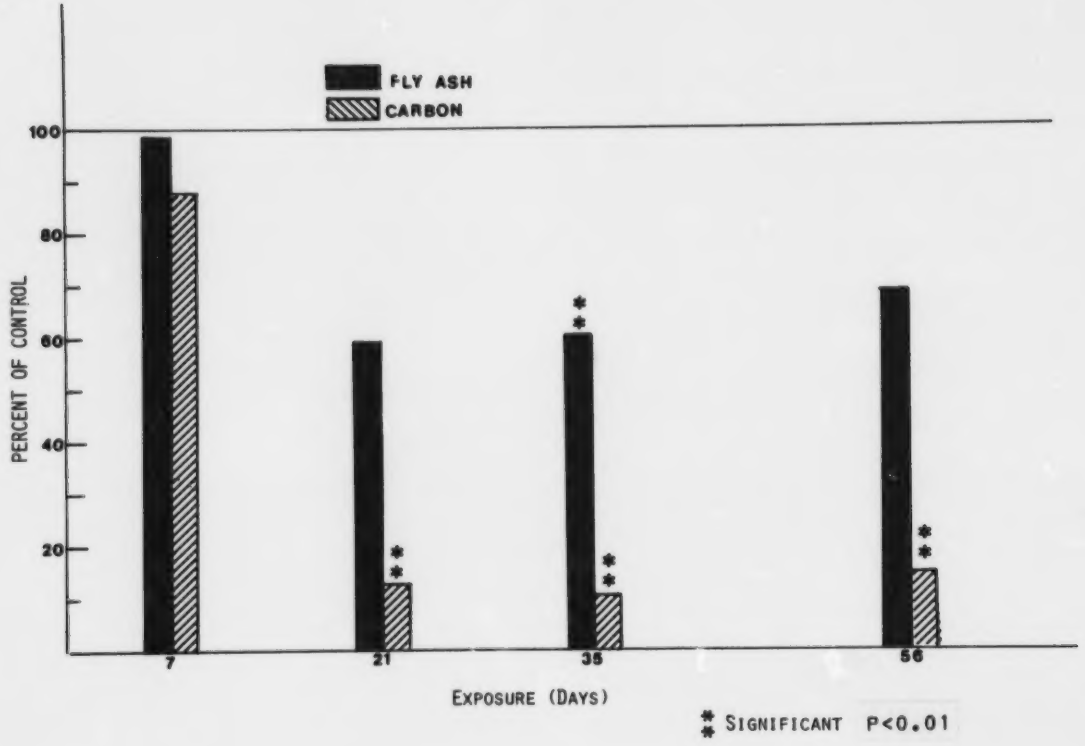


Figure 2. Effects of fly ash and carbon inhalations: Number† of antibody-forming cells/10^6 spleen cells in response to E. coli antigen given as an aerosol.

† values expressed as geometric means

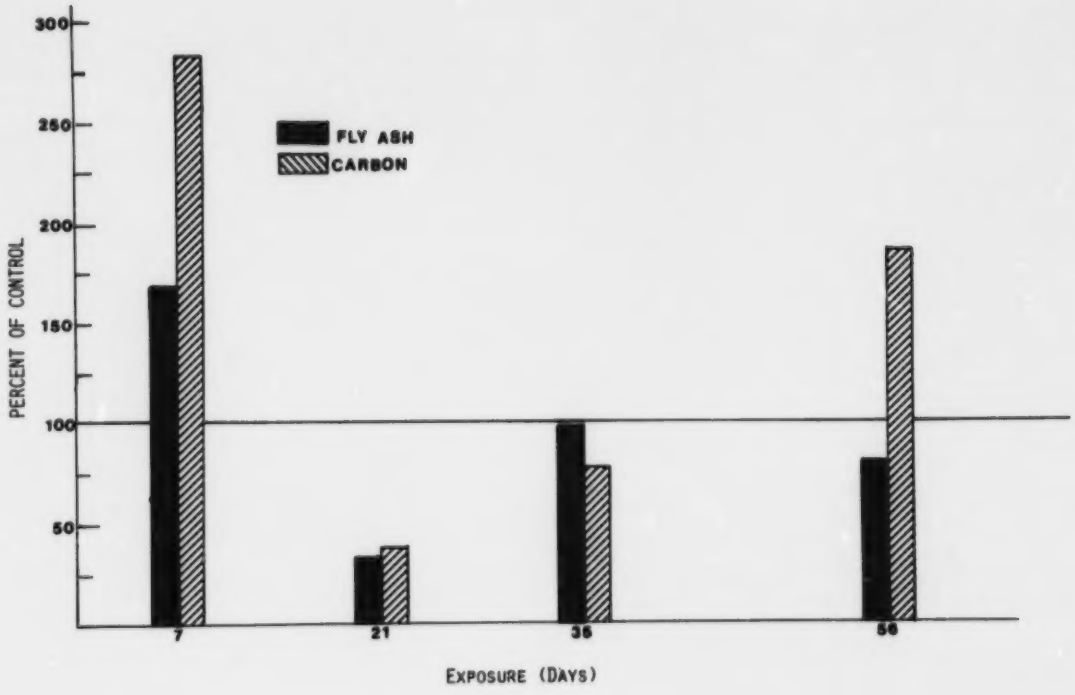


Figure 3. Effects of fly ash and carbon inhalations: Number† of antibody-forming cells/10^6 mediastinal lymph node cells in response to E. coli antigen given as an aerosol.

† values expressed as geometric means

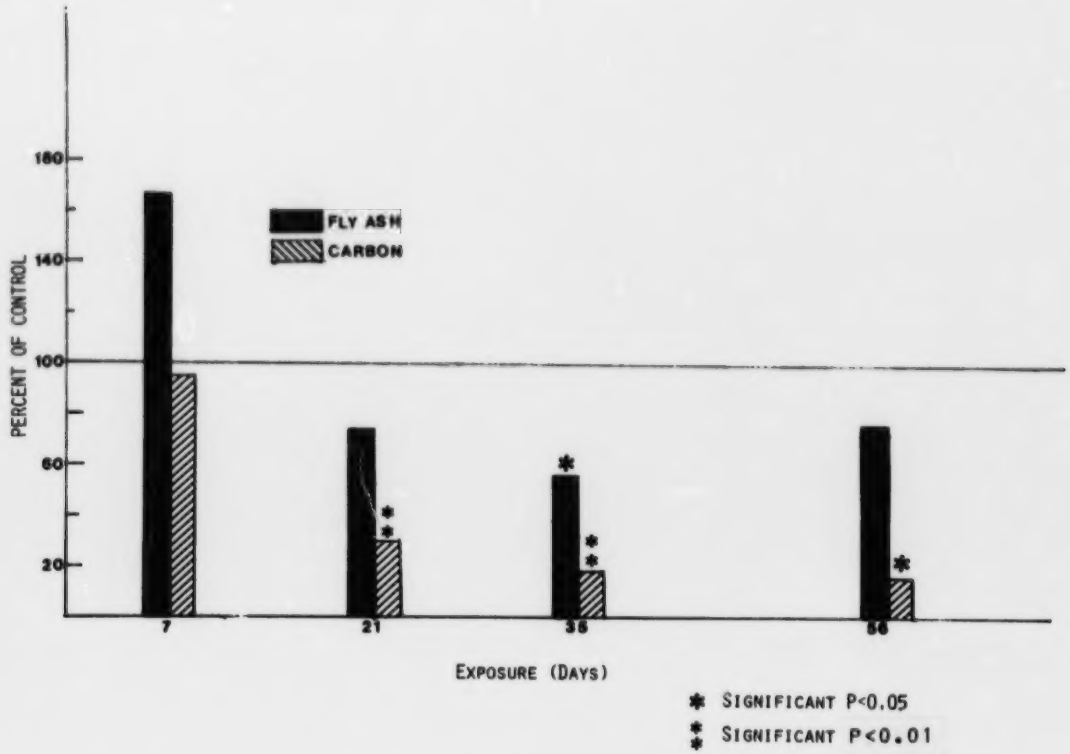


Figure 4. Effects of fly ash and carbon inhalations: Serum agglutinating activity in response to E. coli antigen given as an aerosol.

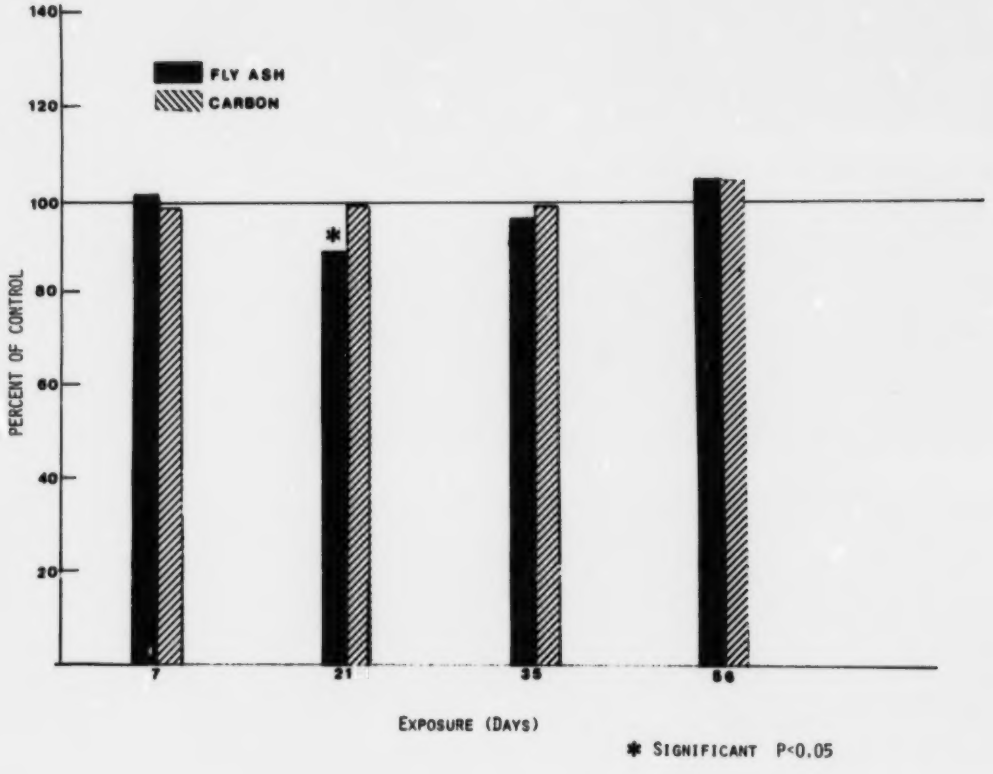


Figure 5. Effects of fly ash and carbon inhalations: ^{3}H - thymidine incorporation into Con A responsive spleen cells.

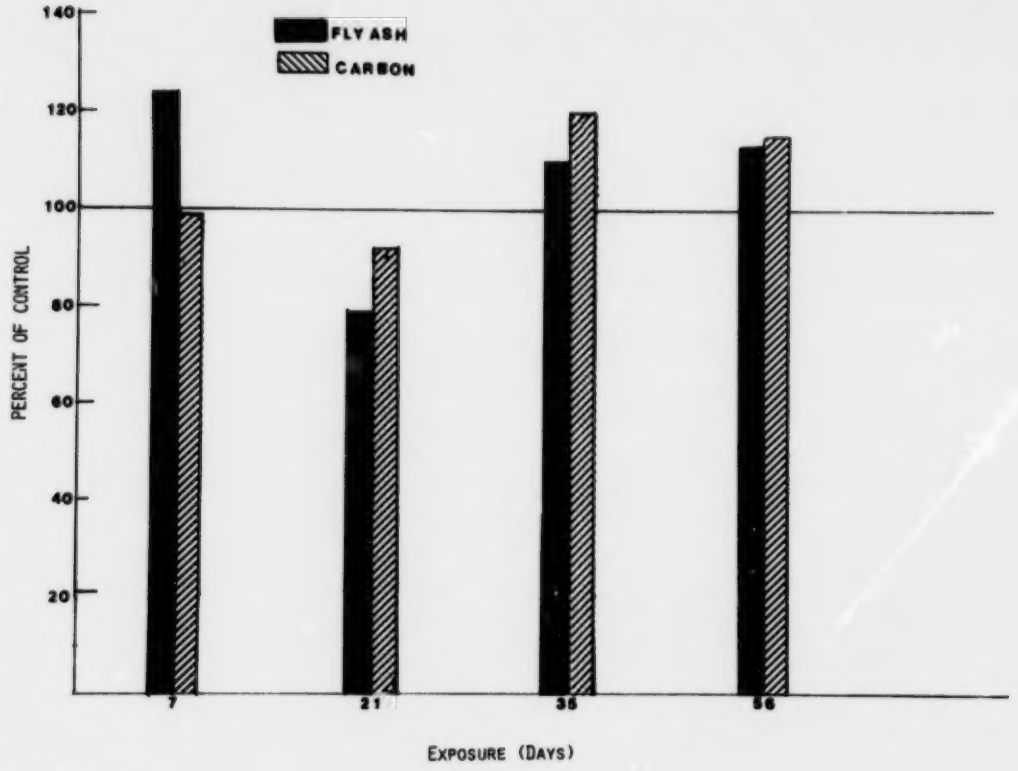


Figure 6. Effects of fly ash and carbon inhalations: ^{3}H - thymidine incorporation into LPS responsive spleen cells.

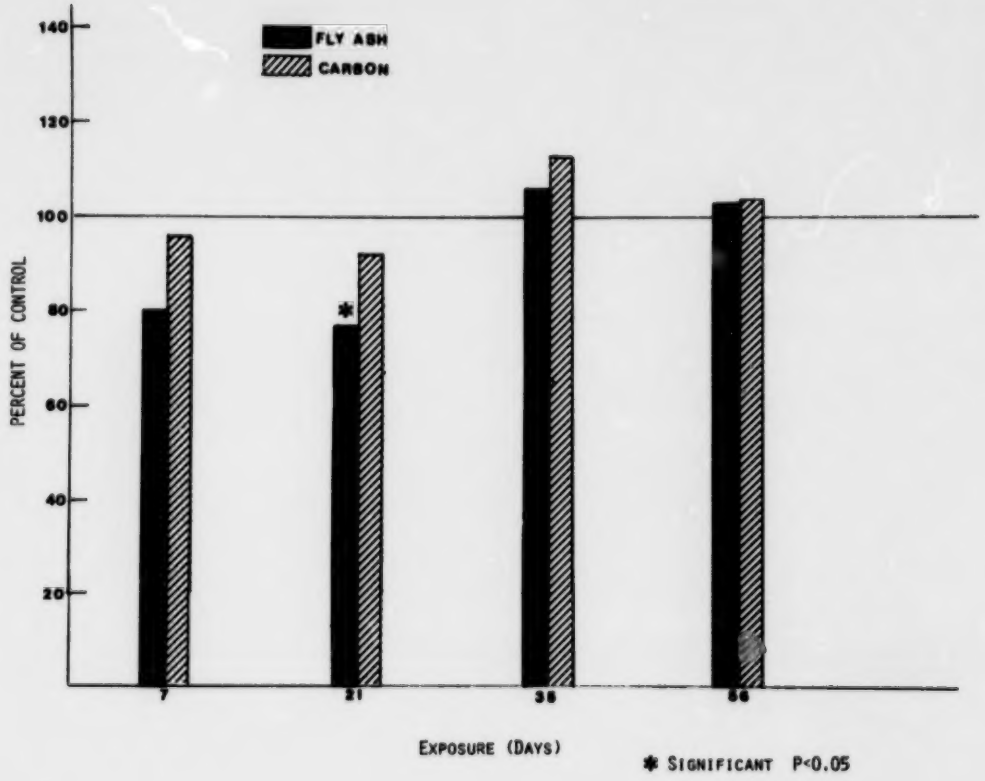


Figure 7. Effects of fly ash and carbon inhalation:
^{3}H - thymidine incorporation into a one-way mixed lymphocyte culture.

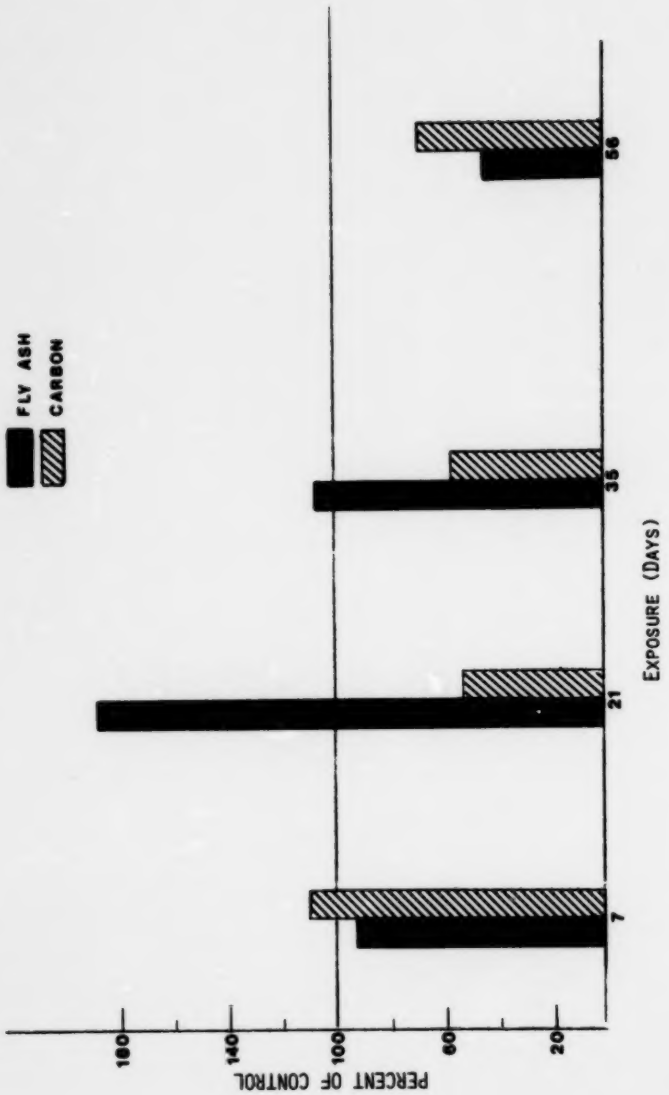


Figure 8. Effects of fly ash and carbon inhalations: cytolytic activity of stimulated T lymphocytes from spleens.

TABLE 1. PARTICULATE EXPOSURES CONCENTRATIONS EXPRESSED AS $\mu g/m^3$ OF AIR
SERIES I EXPERIMENTS

Experiment	Exposure	Ave. fly ash ± S.E.M. Total	Ave. fly ash ± S.E.M. (<2.1 microns)	Ave. carbon black ± S.E.M. Total	Ave. carbon black ± S.E.M. (<2.1 microns)
Responses to Antigenic Stimulations In-Vivo	7 days (7/28-8/4/77)	2667 ± 1417	655 ± 348	4736 ± 1683	947 ± 555
	21 days (7/28-8/18/77)	3357 ± 798	959 ± 236	4987 ± 1334	1387 ± 522
	35 days (7/28-9/2/77)	3220 ± 512	1009 ± 153	4805 ± 855	1561 ± 349
	56 days (7/28-9/22/77)	2658 ± 463	860 ± 140	4615 ± 700	1472 ± 269
Responses to Mitogens In-vitro and Recognitive Activity of T Cells	7 days (8/8-8/15/77)	4976	1433	2461	859
	21 days (8/8-8/30/77)	3686 ± 647	1214 ± 110	4879 ± 1644	1815 ± 560
	35 days (8/8-9/13/77)	3281 ± 440	1130 ± 85	4301 ± 1049	1679 ± 363
	56 days (8/14-10/11/77)	2277 ± 350	840 ± 135	4956 ± 762	1642 ± 296
Cytolytic Activity of Stimulated T Cells	7 days (9/2-9/9/77)	2417	909	2148	924
	21 days (9/2-9/23/77)	1535 ± 563	562 ± 230	4237 ± 1458	1291 ± 485
	35 days (9/2-10/7/77)	1732 ± 444	643 ± 182	4448 ± 1053	1220 ± 350
	56 days (8/19-10/14/77)	2137 ± 380	798 ± 151	4445 ± 670	1449 ± 267

BEGIN LAST LINE OF TEXT
3"/8"
EPA-287 (Cin.) (4-76)
PAGE NUMBER 23
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
DROPPED HEAD; BEGIN SECTIONS HERE
BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA

TABLE 2. RESPONSE TO STIMULATION WITH AEROSOLS OF E.coli 055:B5 ANTIGEN SERIES I EXPERIMENTS

Day	Total number of antibody forming cells/spleen expressed as $\log_{10} \pm$ S.E. Fly ash	Carbon	Control
7	4.059 ± 0.097	4.040 ± 0.090	4.103 ± 0.048
21	3.851 ± 0.055[d]	3.254 ± 0.122[b]	4.146 ± 0.135
35	3.938 ± 0.041[b,d]	3.214 ± 0.140[b]	4.194 ± 0.053
56	3.838 ± 0.056[d]	3.229 ± 0.154[b]	4.026 ± 0.119

Day	Plaque forming units/10^6 spleen cells ± S.E. Fly ash	Carbon	Control
7	1.896 ± 0.112	1.818 ± 0.086	1.874 ± 0.040
21	1.716 ± 0.047[d]	1.047 ± 0.114[b]	1.942 ± 0.128
35	1.842 ± 0.037[b,d]	1.082 ± 0.142[b]	2.061 ± 0.043
56	1.854 ± 0.054[d]	1.180 ± 0.131[b]	2.019 ± 0.104

Day	Hemagglutination titer ($\log_{10}$ +1) ± S.E. Fly ash	Carbon	Control
7	2.553 ± 0.113	2.310 ± 0.071	2.332 ± 0.065
21	2.452 ± 0.074[d]	2.051 ± 0.058[b]	2.588 ± 0.051
35	2.545 ± 0.067[a,d]	1.035 ± 0.184[b]	2.804 ± 0.071
56	2.251 ± 0.058[c]	1.553 ± 0.264[a]	2.393 ± 0.170

[a] Fly ash or carbon compared to control $p < 0.05$.

[b] Fly ash or carbon compared to control $p < 0.01$.

[c] Fly ash compared to carbon $p < 0.05$.

[d] Fly ash compared to carbon $p < 0.01$.

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE

TABLE 3. ANTIBODY RESPONSE OF FLY ASH-EXPOSED MICE TO LPS AEROSOL†

Treatment	PFC/spleen (Log_{10}) ± S.E.	PFC/10^6 spleen cells (Log_{10}) ± S.E.	PFC/10^6 MLN cells (Log_{10}) ± S.E.
Fly Ash	3.0014±0.1788***	0.8321±0.1709***	1.7836±0.2240
Control	4.2017±0.1451	2.0625±0.1469	2.0219±0.2151

† Mice were exposed to fly ash for 9 months at an average respirable dust concentration of 2189 µg/m^3 of air. The aerosol contained 10 mg of LPS.

*** $p < 0.001$.

TABLE 4. ANTIBODY RESPONSE OF FLY ASH-EXPOSED MICE TO INTRATRACHEAL INJECTION OF LPS†

Treatment	PFC/spleen (Log_{10}) ± S.E.	PFC/10^6 spleen cells (Log_{10}) ± S.E.	PFC/10^6 MLN cells (Log_{10}) ± S.E.
Fly Ash	4.3031±0.1193**	2.1014±0.1373**	1.7760±0.1123
Control	4.9377±0.0931	2.6934±0.0860	2.2132±0.1955

† Mice were exposed to fly ash for 9 months at an average respirable dust concentration of 2189 µg/m^3 of air. They were then injected intratracheally with 50 µg LPS.

** $p < 0.01$.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

TABLE 5. LYMPHOCYTE TRANSFORMATIONS - SERIES I
DISINTEGRATIONS PER MINUTE ± S.E.

Treatments			
Concanavilin A	**Fly ash**	**Carbon**	**Control**
7 day	376480 ± 8072	364254 ± 10599	371294 ± 7149
21	243584 ± 10726	272099 ± 10551	274816 ± 8765
35	344347 ± 4629	357967 ± 6783	362403 ± 7755
56	312711 ± 1842	312288 ± 3098	299615 ± 12,842
Lipopolysaccharide			
7	38248 ± 5417	30495 ± 4023	30857 ± 2028
21	19279 ± 2222	22465 ± 2923	24437 ± 3289
35	28843 ± 3058	31531 ± 2003	26338 ± 2681
56	45866 ± 4126	46597 ± 1606	40475 ± 5239
Mixed Lymphocyte Reaction			
7	17684 ± 1110	21301 ± 2471	22130 ± 2001
21	15703 ± 1134[a]	18688 ± 1522	20318 ± 1304
35	30235 ± 1378	32161 ± 2157	28427 ± 1312
56	18878 ± 696	19061 ± 437	18349 ± 1242
Control 48h			
7	12313 ± 600	14495 ± 525	17115 ± 1421
21	12614 ± 160	12403 ± 490	14319 ± 952
35	11348 ± 571	10574 ± 1109	10867 ± 1005
56	17623 ± 1031	14682 ± 1079	14606 ± 998
Control 72h			
7	12405 ± 627	12736 ± 700	12940 ± 977
21	11433 ± 725[a]	14057 ± 1261	15639 ± 1266
35	14019 ± 802	13891 ± 906	14749 ± 1358
56	20524 ± 885[c]	17624 ± 581	18153 ± 1133

[a] Fly ash or carbon compared to control $p < 0.05$.

[b] Fly ash or carbon compared to control $p < 0.01$.

[c] Fly ash compared to carbon $p < 0.05$.

[d] Fly ash compared to carbon $p < 0.01$.

TABLE 6. LYMPHOCYTE TRANSFORMATIONS
SERIES I EXPERIMENTS

Treatments	Stimulation index ± S.E.			
Concanavilin A	Fly ash	Carbon	Control	
7 day	31.2 ± 1.9[a,c]	25.3 ± 0.9	22.9 ± 2.2	
21	19.4 ± 1.0[c]	22.1 ± 0.8	20.0 ± 1.7	
35	30.9 ± 1.6	36.4 ± 3.6	34.7 ± 2.2	
56	18.3 ± 1.3	21.9 ± 1.4	20.9 ± 3.3	
Lipopolysaccharide				
7	3.2 ± 0.5	2.4 ± 0.3	2.5 ± 0.3	
21	1.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.3	
35	2.1 ± 0.1	2.3 ± 0.1	1.8 ± 0.1	
56	2.2 ± 0.1	2.7 ± 0.2	2.2 ± 0.2	
Mixed lymphocyte reaction				
7	1.5 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	
21	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	
35	2.2 ± 0.2	2.5 ± 0.3	2.1 ± 0.2	
56	0.9 ± 0.0	1.1 ± 0.0	1.0 ± 0.0 →	No apparent stimulation

[a] Fly ash or carbon compared to control $p < 0.05$.

[b] Fly ash or carbon compared to control $p < 0.01$.

[c] Fly ash compared to carbon $p < 0.05$.

[d] Fly ash compared to carbon $p < 0.01$.

TABLE 7. BODY AND SPLEEN WEIGHTS OF MICE USED FOR LYMPHOCYTE TRANSFORMATIONS ± S.E.
SERIES I EXPERIMENTS

body weight (g)	Fly ash	Carbon	Control
7 days	22.0 ± 0.3	21.4 ± 0.6	22.6 ± 0.8
21	19.0 ± 0.9	18.8 ± 0.8	19.7 ± 0.9
35	19.9 ± 0.6	19.9 ± 0.5	19.9 ± 0.6
56	21.8 ± 0.6	21.7 ± 0.2	21.7 ± 0.4
Spleen weight (g)			
7 days	0.116 ± 0.004	0.117 ± 0.006	0.135 ± 0.009
21	0.140 ± 0.006	0.122 ± 0.008	0.124 ± 0.004
35	0.110 ± 0.005	0.113 ± 0.017	0.112 ± 0.011
56	0.107 ± 0.005	0.110 ± 0.003	0.124 ± 0.007

[a] Fly ash or carbon compared to control $p < 0.05$.

[b] Fly ash or carbon compared to control $p < 0.01$.

[c] Fly ash compared to carbon $p < 0.05$.

[d] Fly ash compared to carbon $p < 0.01$.

TABLE 8. CYTOLYTIC ACTIVITY OF SPLEEN CELLS EXPRESSED AS LOG_{10} NUMBER OF SPLEEN CELLS NEEDED FOR 10% LYSIS ± S.E. SERIES I EXPERIMENTS

Day	Fly ash	Carbon	Control
7	5.720 ± 0.080	5.646 ± 0.123	5.687 ± 0.046
21	4.913 ± 0.063[c]	5.463 ± 0.099	5.187 ± 0.107
35	4.817 ± 0.060	5.086 ± 0.164	4.846 ± 0.168
56	5.254 ± 0.164	5.064 ± 0.124	4.898 ± 0.155

[a] Fly ash or carbon compared to control $p < 0.05$.

[b] Fly ash or carbon compared to control $p < 0.01$.

[c] Fly ash compared to carbon $p < 0.05$.

[d] Fly ash compared to carbon $p < 0.01$.

TABLE 9. NUMBER OF ANTIBODY FORMING CELLS IN POOLED MEDIASTINAL LYMPH NODES/10^6 LYMPH NODE CELLS RESPONSE TO STIMULATION WITH *E.coli* ANTIGEN SERIES I EXPERIMENTS

Exposure	Response in M.L.N/10^6 cells Fly ash	Carbon	Control
7	28.0	46.8	16.5
21	32.7	38.6	101.8
35	28.5	22.4	28.7
56	59.9	138.8	74.8

PART II

The second series of experiments was done in order to confirm the results of the first set of studies and to compare the effects obtained using PSU obtained fly-ash with fly-ash provided by EPA. The procedures used to determine the effects of fly-ash and carbon in the second series included studies of pulmonary macrophage phagocytic activity.

Mice, in groups of eight, were exposed to PSU fly-ash, EPA fly-ash and carbon. The total concentration of these particulates and proportions that were smaller than 2.1 μ (respirable range) are shown in Table 10. This table also lists the length of the exposure periods.

The effects of these exposures on mice are shown in Tables 11-17 and Figures 9-16. The effect of fly-ash on humoral responses (antibody forming cells and serum agglutinating activity) was much less pronounced than that of equivalent time exposures of carbon dust. Though the concentrations of fly ash were lower than those of carbon, due to a greater difficulty in dispersing fly ash, the pattern of progressively more pronounced suppression with time which occurred after carbon exposures was not found after fly ash exposures. A decreased number of antibody forming cells in the spleens occurred after 7 and 24 days of exposure to fly ash, and decreased serum antibody activity was found after 7, 21 and 56 days of exposure (Figures 9, 10, 12). Carbon dust caused a very pronounced and progressive reduction in the number of antibody forming cells in the spleens after all times of exposure, with almost complete suppression after 148 days of exposure (Figures 9, 10). The effects on the mediastinal lymph nodes varied from enhancement in the number of antibody forming cells produced by fly ash after 7 and 21 days of exposure to no effect or a small decrease of response after 56 days.

The cellular immune reactions were not affected in most cases. The one exception was suppression of cytolytic activity by PSU fly ash after 35 days of exposure (Figure 15).

Phagocytic activity by lung derived macrophages was reduced by both fly ash and carbon starting at 21 days of exposure with a progressive decrease of phagocytosis up to 146 days of exposure (Figure 16).

TABLE 10. CONCENTRATION OF PARTICULATES DURING THE SERIES II EXPERIMENTS EXPRESSED AS $\mu g/m^3$ OF AIR

	Exposure	Ave. fly ash ± S.E.M. Total	Ave. fly ash ± S.E.M. (<2.1 microns)	Ave. carbon black ± S.E.M. Total	Ave. carbon black ± S.E.M. (<2.1 microns)
In-vivo Responses to Antigenic Stimulations	7 days (11/16-11/23/77)	2232±256	933±77	4492±1917	1538±1029
	21 days (11/18-12/9/77)	2039±343	785±139	4707±1192	1472±386
	21 days (11/23-12/14/77)	2143±286	764±130	3608±355	1104±125
	35 days (12/8/77-1/12/78)	2059±289	741±86	2511±346	857±157
		*1628±375	433±77		
	56 days (12/15/77-2/9/78)	1502±285	543±100	2934±459	841±125
		*2634±430	615±98		
	148 days (12/22/77-5/19/78)	1459±140	535±50	3509±312	1178±119
		*1831±221	461±50		
In-vitro Responses to Mitogens, Recognitive Activity of T cells and Phagocytosis by Macrophages	7 days (12/8-12/15/77)	2262±298	794±104	3180±550	932±161
		*1159±228	366±72		
	21 days (11/15-12/6/77)	2042±343	809±136	5399±1372	1407±429
	35 days (11/16-12/21/77)	2115±235	804±90	4027±910	1248±292
	56 days (1/10-3/7/78)	1334±237	502±86	2984±512	885±139
		*2674±97	623±97		
	148 days (12/13/77-5/9/78)	1297±131	509±48	3520±342	1162±130
		1879±240	465±55		
Cytolytic Activity of Stimulated T cells	7 days (1/13/77-1/20/78)	1905±387	661±134	3758±1933	996±512
		*3129±1281	691±283		
	35 days (11/29/77-1/3/78)	2113±384	703±117	3172±526	909±142
		*2819±839	676±194		
	56 days (1/11-3/8/78)	1134±237	502±86	2984±512	885±139
		*2674±429	623±96		
	154 days (12/20-5/23/78)	1459±140	535±50	3509±312	1178±119
		*1831±221	461±50		

* Exposure data for E.P.A. supplied fly ash.

TABLE 11. RESPONSE OF MICE TO STIMULATIONS WITH AEROSOL OF E.coli 055:B5 ANTIGEN:

SERIES II EXPERIMENTS

Total number of antibody forming cells in spleens (log_{10}) ± S.E.

Day	Fly ash group	Carbon group	Control group
7	4.059 ± .114[d]	3.632 ± .072[b]	4.054 ± .040
21	3.553 ± .105[a]	3.511 ± .035[b]	3.910 ± .070
21 (LPS)	5.042 ± .030	5.059 ± .114	5.071 ± .076
35	3.848 ± .096[c]	3.469 ± .096[a]	3.818 ± .122
	*4.0169± .039[d]		
56	3.798 ± .155[d]	2.903 ± .167[b]	3.824 ± .068
	*3.798 ± .105[d]		
148	3.995 ± .091[d]	2.5453± .081[b]	4.030 ± .101
	*3.970 ± .122[d]		

Plaque forming units/10^6 spleen cells (log_{10}) ± S.E.

Day	Fly ash group	Carbon group	Control group
7	1.925 ± .130[c]	1.575 ± .041[b]	1.894 ± .042
21	1.605 ± .096[a]	1.543 ± .048[b]	1.889 ± .074
21 (LPS)	2.900 ± .062	2.846 ± .111	2.852 ± .070
35	1.753 ± .082[c]	1.386 ± .096[a]	1.754 ± .111
	*1.901 ± .032[d]		
56	1.615 ± .161[d]	0.899 ± .135[b]	1.809 ± .055
	*1.719 ± .107[d]		
148	1.825 ± .080[d]	0.343 ± .079[b]	1.813 ± .098
	*1.765 ± .110[d]		

Hemagglutination titers (log_{10} ±1) S.E.

Day	Fly ash group	Carbon group	Control group
7	2.647 ± .132[c]	2.233 ± .109[a]	2.706 ± .116
21	1.812 ± .122[b]	1.864 ± .111[b]	2.425 ± .070
35	2.829 ± .143	2.513 ± .090	2.798 ± .133
	*2.955 ± .106		
56	3.067 ± .168	2.474 ± .094	3.475 ± .070
	*3.045 ± .081		
148	2.754 ± .100[d]	2.108 ± .071[b]	2.670 ± .112
	*2.676 ± .103[d]		

* E.P.A. supplied fly ash.

[a] Fly ash or carbon group compared to control group, difference significant at $p < 0.05$.

[b] Fly ash or carbon group compared to control group, difference significant at $p < 0.01$.

[c] Fly ash group compared to carbon group, difference significant at $p < 0.05$.

[d] Fly ash group compared to carbon group, difference significant at $p < 0.01$.

TABLE 12. LYMPHOCYTE TRANSFORMATION - SERIES II (SPLEEN CELLS)
DISINTEGRATIONS PER MINUTE + S.E.

Treatments	PSU Fly ash	EPA Fly ash	Carbon	Control
Concanavilin A				
7 day	382980±8133	386251±6217	386770±4604	377058±5250
21	455857±3987	-	458208±4557	447051±12015
35	189347±17793	-	228674±4557	226212±3951
56	447784±8161	447527±11121	444658±5372	455863±10586
148	361164±14468[c]	391681±9454	410621±6528	381916±11765
Lipopolysaccharide				
7	45150 ±5533	43610 ±3413	45451 ±3659	50053 ±2020
35	25698 ±5069	-	37481 ±2372	35633 ±2589
56	85586 ±3398	77074 ±3334	78691 ±4308	81792 ±3242
148	95193 ±9940	103948±5949	106371±9576	107308±4616
Control 48h				
7	11941 ±1085	12360 ±545	17068 ±3413	13581 ±1159
21	16010 ±713	-	17479 ±1202	15985 ±1095
35	9446 ±1519	-	12479 ±544	11064 ±498
56	35793 ±2112	34167 ±2799	34338 ±2451	34873 ±1599
148	21639 ±1165[c]	25404 ±1261	26455 ±907	23483 ±1364
Control 72h				
7	18421 ±1646	16661 ±565[a]	20349 ±2250	21294 ±1386
21	-	-	-	-
35	17025 ±2626	-	25171 ±1588	22961 ±1194
56	49479 ±2844	45564 ±3022	47738 ±2577	48504 ±1648
148	38335 ±1973	41887 ±1621	42510 ±1673	41434 ±2294

[a] Fly ash or carbon compared to control $p < 0.05$.

[b] Fly ash group compared to carbon group $p < 0.05$.

TABLE 13. LYMPHOCYTE TRANSFORMATIONS - SERIES II (SPLEEN CELLS)
STIMULATION INDEX + S.E.

Treatments	PSU Fly ash	EPA Fly ash	Carbon	Control
Concanavalin A				
7 day	34.7 ± 4.5	31.6 ± 1.3	27.3 ± 3.7	29.1 ± 2.2
21	28.9 ± 1.4	-	27.7 ± 1.9	29.4 ± 3.2
35	22.6 ± 2.4	-	18.6 ± 0.7	20.8 ± 1.1
56	12.9 ± 1.0	13.8 ± 1.2	13.4 ± 0.9	13.2 ± 0.5
148	17.0 ± 0.9	15.8 ± 1.0	15.6 ± 0.6	17.0 ± 0.9
Lipopolysaccharide				
7	2.5 ± 0.3	2.7 ± 0.3	2.4 ± 0.2	2.4 ± 0.2
35	1.5 ± 0.1	-	1.5 ± 0.1	1.6 ± 0.1
56	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
148	2.5 ± 0.3	2.5 ± 0.2	2.5 ± 0.2	2.6 ± 0.1

TABLE 14. NUMBER OF ANTIBODY FORMING CELLS IN POOLED MEDIASTINAL LYMPH NODES/10^6 LYMPH NODE CELLS

	Response to stimulations with *E. coli* antigens		
Day	Fly ash group	Carbon group	Control group
7	76.9	33.9	31.6
21	39.0	28.5	15.8
35	29.0	18.8	23.3
	* 26.9		
56	56.9	28.9	32.8
	* 17.3		
148	33.3	48.9	32.4
	* 25.5		

* E.P.A. supplied fly ash.

TABLE 15. SPLEEN WEIGHTS IN GRAMS ± S.E.
SERIES II EXPERIMENTS

Day	Fly ash group	Carbon group	Control group
7	0.160 ± .010[c]	0.140 ± .009[a]	0.189 ± .014
21	0.105 ± .009	0.098 ± .004[a]	0.111 ± .004
35	0.102 ± .005	0.098 ± .006	0.103 ± .003
	* 0.108 ± .004		
56	0.106 ± .006	0.096 ± .007	0.098 ± .009
	* 0.113 ± .007		
148	0.104 ± .003	0.109 ± .004	0.113 ± .005
	* 0.111 ± .006		

* Data for E.P.A. supplied fly ash.

[a] Fly ash or carbon group compared to control group, difference significant at $p < 0.05$.

[c] Fly ash group compared to carbon group, difference significant at $p < 0.05$.

TABLE 16. CYTOLYTIC ACTIVITY OF SPLEEN CELLS EXPRESSED AS LOG_{10} NUMBER OF SPLEEN CELLS NEEDED FOR 10% LYSIS ± S.E. SERIES II EXPERIMENTS

Day	Fly ash group	Carbon group	Control group
7	5.002 ± .217	4.836 ± .114	5.067 ± .237
	* 4.996 ± .128		
35	5.524 ± .197[a]	5.139 ± .099	4.971 ± .117
	* 5.130 ± .194		
56	Insufficient amount of cytolytic activity for determination of 10% lysis		
154	5.876 ± .120	5.384 ± .132	5.630 ± .150
	* 5.733 ± .153		

* E.P.A. supplied fly ash.

[a] Fly ash or carbon group compared to control group, difference significant at $p < 0.05$.

TABLE 17. IN VITRO PHAGOCYTOSIS OF *E. coli* BACTERIA BY ALVEOLAR MACROPHAGES: PER CENT OF MACROPHAGES CONTAINING *E. coli* ± S.E.

Day	Fly ash group	Carbon group	Control group
7	25.56 ± 2.41	19.50 ± 2.22	20.75 ± 1.03
	* 19.36 ± 1.58		
21	16.92 ± 1.64[c]	12.34 ± 1.33[b]	22.61 ± 2.42
35	8.54 ± 1.53[a]	6.94 ± .90[b]	13.31 ± 1.03
56	24.94 ± 2.68[b,d]	16.38 ± 2.08[a]	36.94 ± 2.30
	* 29.31 ± 1.16[a,d]		
148	36.57 ± 2.96[b,d]	24.19 ± 1.91[a]	54.56 ± 1.97
	* 35.57 ± 3.13[b,d]		

* E.P.A. fly ash.

[a] Fly ash or carbon group compared to control group, difference significant at $p < 0.05$.

[b] Fly ash or carbon group compared to control group, difference significant at $p < 0.01$.

[c] Fly ash group compared to carbon group, difference significant at $p < 0.05$.

[d] Fly ash group compared to carbon group, difference significant at $p < 0.01$.

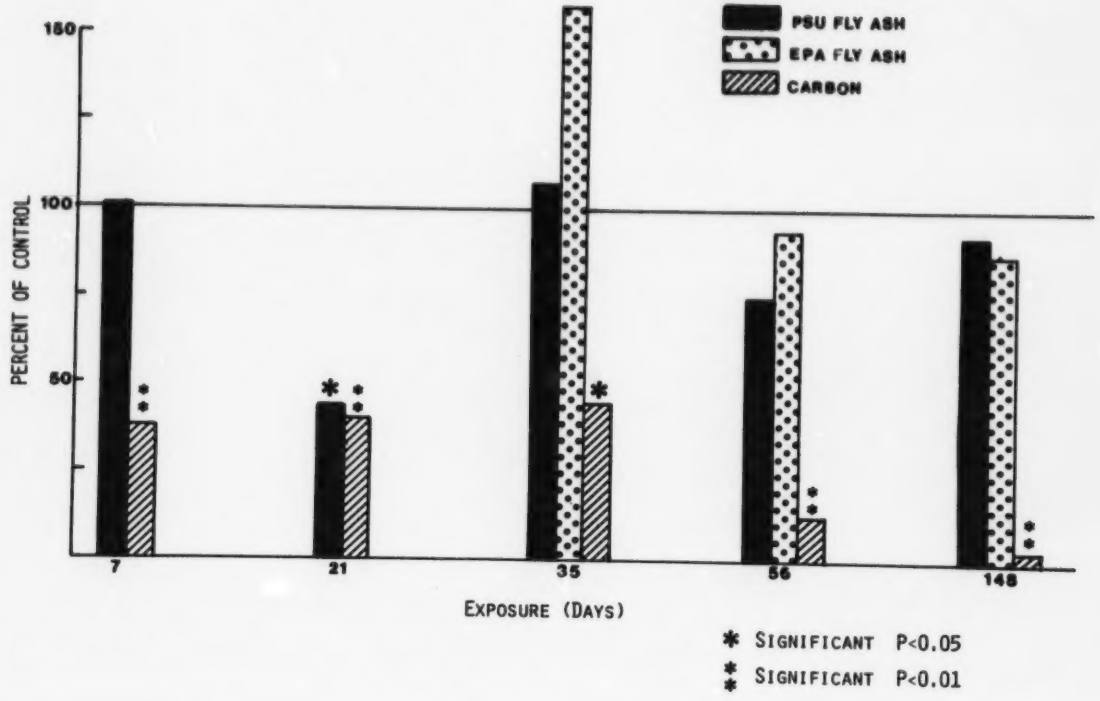


Figure 9. Effects of fly ash and carbon inhalations: Number† of antibody-forming cells in spleens in response to E. coli antigen given as an aerosol. Series II experiments

† values expressed as geometric means

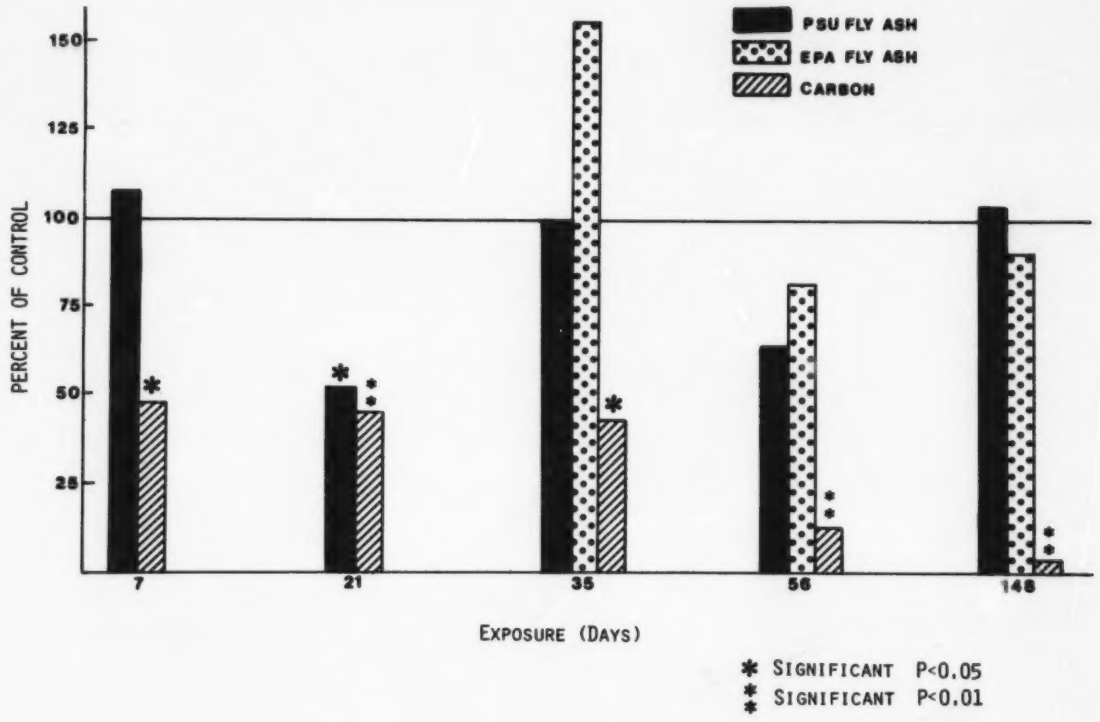


Figure 10. Effects of fly ash and carbon inhalations: Number† of antibody-forming cells/10^6 spleen cells in response to E. coli antigen given as an aerosol. Series II experiments

† values expressed as geometric means

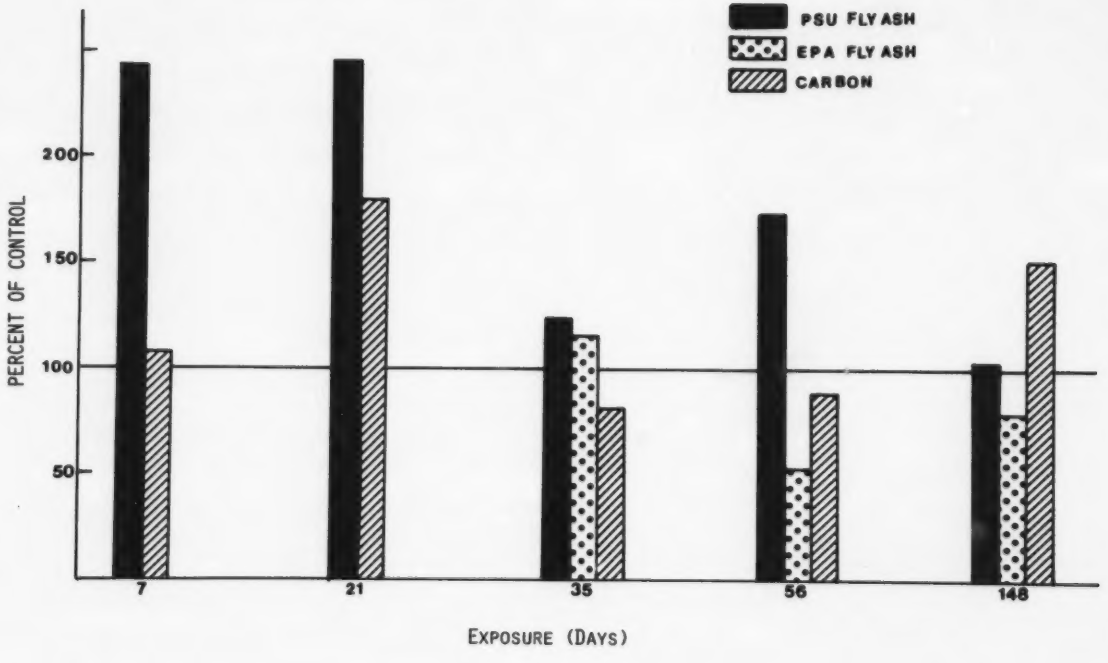


Figure 11. Effects of fly ash and carbon inhalations: Number† of antibody-forming cells/10^6 mediastinal lymph node cells in response to E. coli antigen given as an aerosol. Series II experiments

† values expressed as geometric means

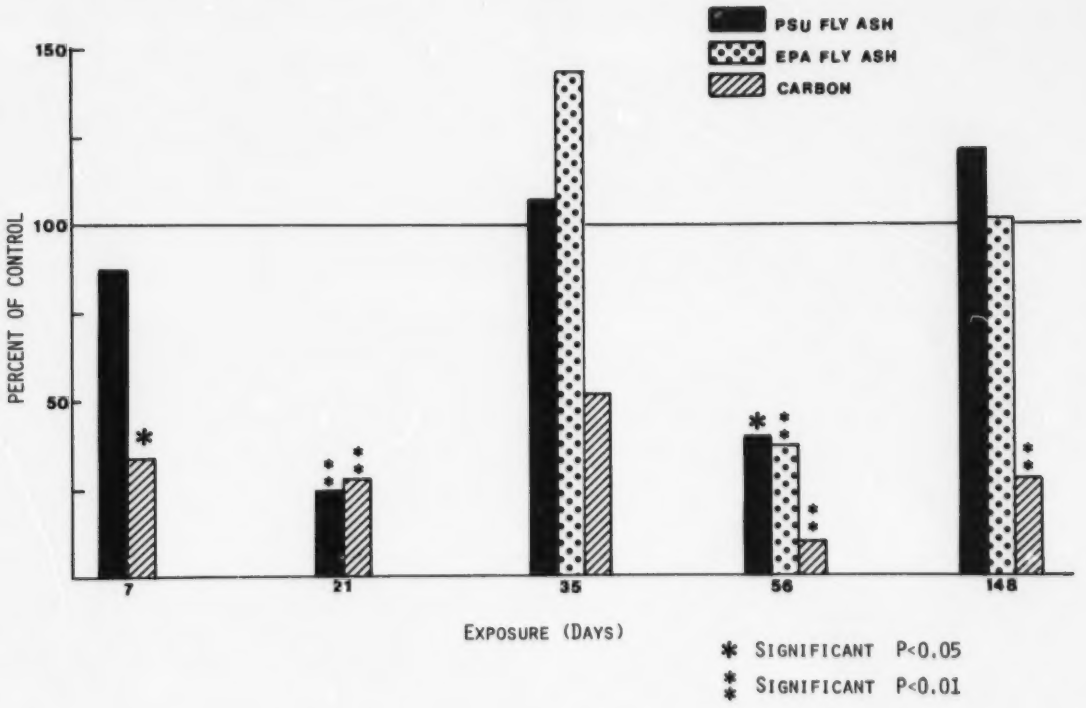


Figure 12. Effects of fly ash and carbon inhalations: Serum agglutinating activity in response to E. coli antigen given as an aerosol. Series II experiments

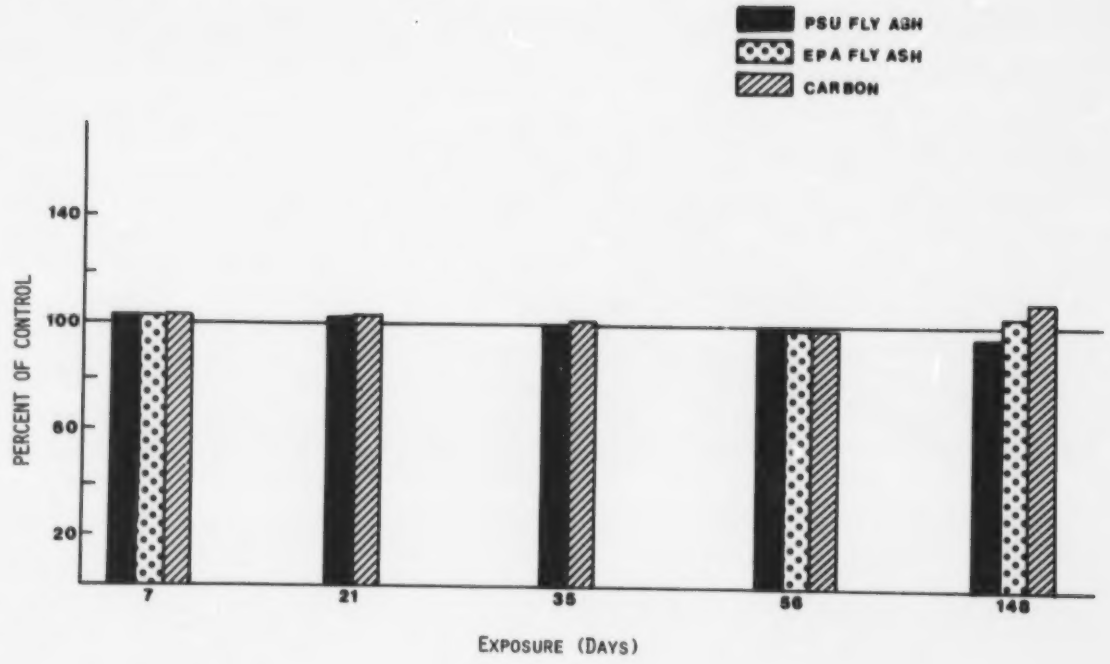


Figure 13. Effects of fly ash and carbon inhalation: ^{3}H - thymidine incorporation into ConA responsive spleen cells. Series II experiments

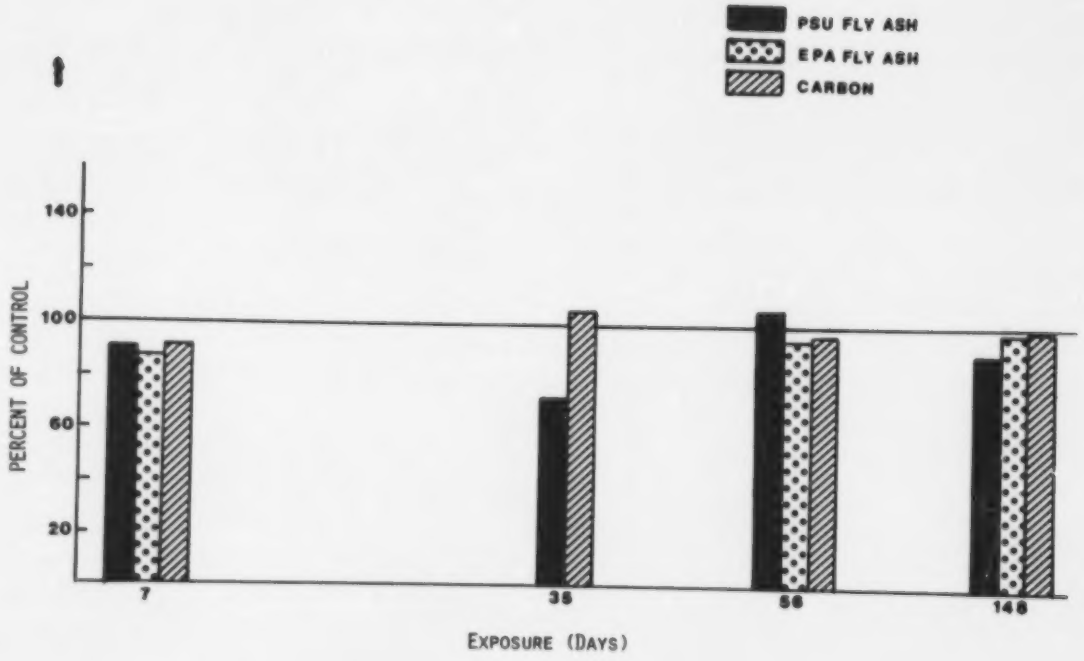


Figure 14. Effects of fly ash and carbon inhalation: ^{3}H - thymidine incorporation into LPS responsive spleen cells. Series II experiments

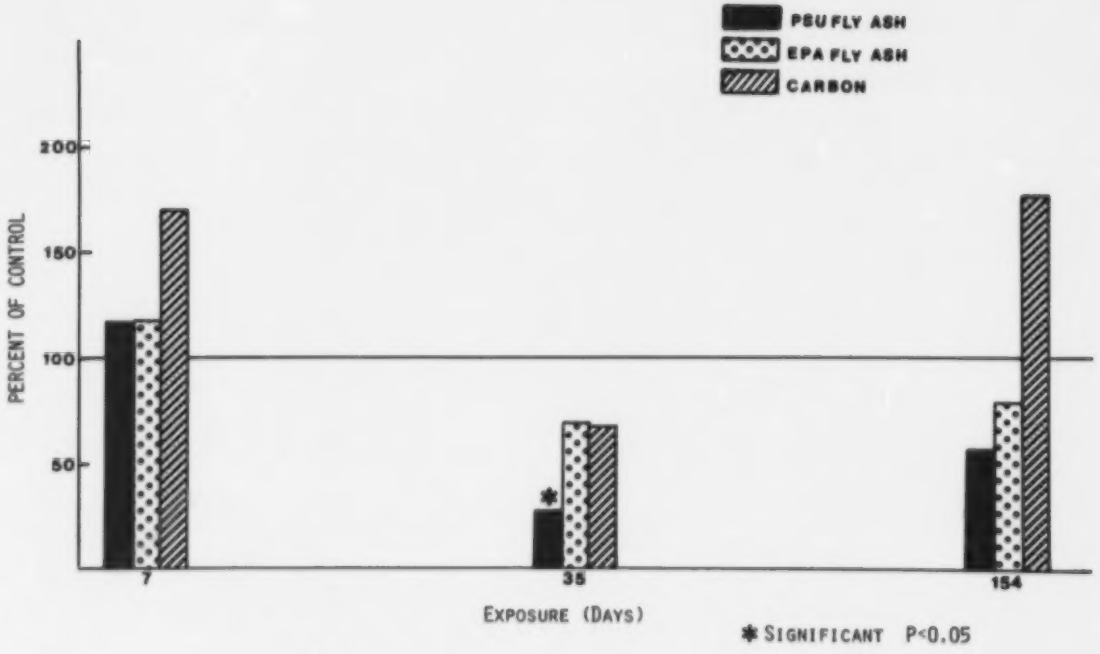


Figure 15. Effects of fly ash and carbon inhalation: Cytolytic activity of stimulated T lymphocytes from spleens. Series II experiments

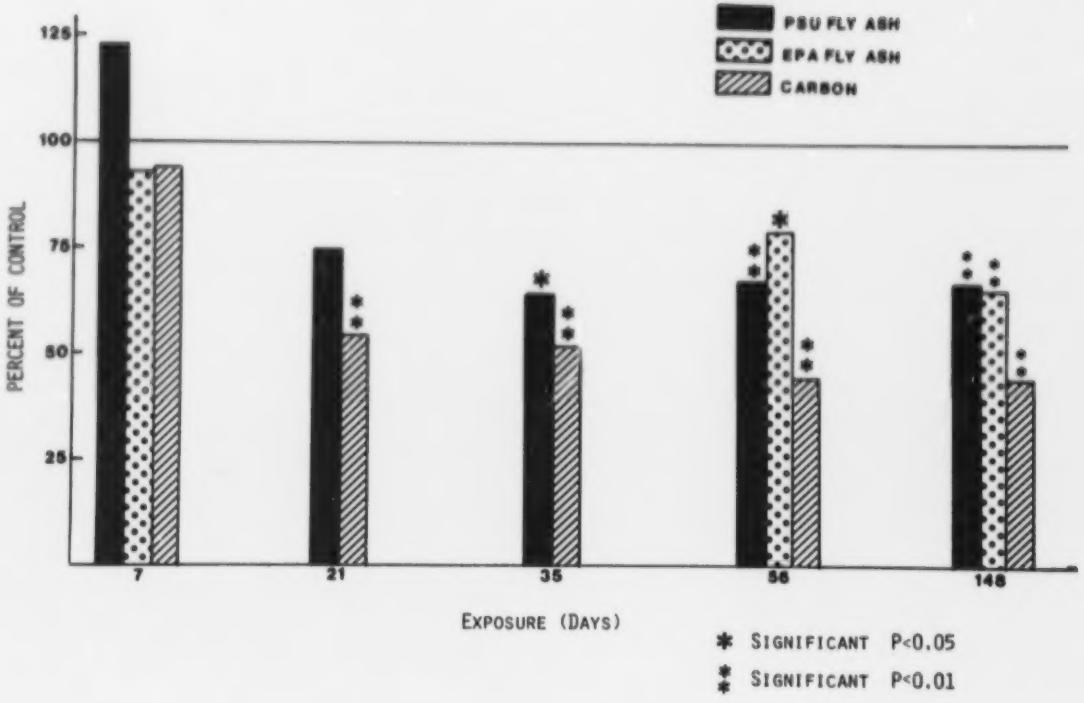


Figure 16. Effects of fly ash and carbon inhalation: In - vitro phagocytosis of E. coli bacteria by pulmonary macrophages. Series II experiments

PART III

The third series of experiments were designed to determine the effects of fly ash on various functions of the alveolar macrophage.

1. Effect of PSU Fly-Ash on Phagocytic Activity of Macrophages

Mice were exposed to fly ash for up to 4 weeks at concentrations of 742 $\mu g/m^3$ of air of particles <2.1μ. These animals were removed at weekly intervals and alveolar macrophages were obtained by teasing the lungs. These were placed in Leighton tubes and incubated for one hour. Following incubation, $2.5X10^7$ Staphylococcus aureus cells were added to the culture tube followed by 0.5 hour incubation period. The media containing unphagocytized bacteria was aspirated off and lysostaphin was added. Macrophages were then stained and using a light microscope their ability to phagocytize S. aureus was determined. The results were tabulated as the percentage of macrophages phagocytizing a certain number of bacteria. These results are shown in Table 18 and indicate that fly ash exposure decreases the proportion of cells capable of phagocytosis and also decreases the proportion of very active macrophages (7 or more bacteria phagocytized).

2. Effect of Fly-Ash on Cell-Mediated Cytotoxicity (ADCC) by Alveolar Macrophages

The initial phase of these studies was to develop the technique for determining the lytic activity of alveolar macrophages obtained from mice, guinea pigs and hamsters. Though satisfactory antibody-dependent lytic activity was present in mouse-derived alveolar macrophages the very low numbers of cells obtained per individual necessitated the pooling of cells from several lungs making the procedure impractical in this species.

Using alveolar cell populations obtained by lung lavage we found the best ADCC against chicken red blood cells (CRBC) in mice after intratracheal injections with BCG and followed by inhalations of PPD (Figure 17). Using this method we found considerable ADCC at several target:effector cell ratios in alveolar cells obtained from both guinea-pigs and golden hamsters, (Figures 18, 19). The high responses of hamster alveolar cells prompted our adoption of the golden hamster as the model in the ADCC studies.

We next attempted to find out if intratracheal exposure to an environmental toxin, such as silica, would change the ADCC effector cell function. ADCC of hamster alveolar cells was determined after intratracheal injection of 2 mg. Minusil at effector to target ratios of 1:1 (Figure 20). Animals were assayed 7 and 14 days post-injection. A slight, but not significant enhancement of ADCC response was found, however the effector to target ratio

was only 1:1. We then decided to repeat this experiment at 14 days post-injection of Minusil using a more optimal effector to target ratio of 10:1. ADCC of hamster lung cells after injection of 2 mg. of Minusil at effector to target ratio of 10:1 was found to be significantly enhanced ($p<0.001$) compared to controls and this ratio was chosen for further experiments.

We then determined what effect intratracheal injection (IT) of an environmental toxin would have on ADCC at various times after injection. IT injections of 2 mg of silica into Syrian golden hamsters significantly enhanced the ADCC 1,7,14,42 and 70 days post injection (Figure 21). When 2 mg of fly ash was used a significant enhancement was found at 14, 42 and 70 days (Figure 22).

In order to determine if silica or fly-ash inhalation affects ADCC, hamsters were exposed to the dusts in chambers and removed for the ADCC determinations after several periods of exposure. Inhalations of respirable size silica at an average concentration of 3102 $\mu g/m^3$ of air caused a slight enhancement of ADCC function after 7 days of exposure and significant enhancement after 14, 42, and 70 days (Figure 23). Inhalation of fly ash (5886 $\mu g/m^3$) did not change ADCC after 7 and 14 days but caused significant suppression after 42 days and 70 days (Figure 24).

In addition ADCC we also began to study the effects of inhaled dust on AM mediated cytotoxicity. IT injection of 2 mg of silica in BCG-primed hamsters was found to activate AM to become tumoricidal against hamsters H50 tumor cells, while IT injections of silica or BCG-priming alone did not cause activation (Figure 25). IT injections of fly ash was found to activate AM to become cytotoxic against H50 tumor cells to an even greater degree than silica (Figure 25).

3. Effect of Fly Ash Exposed Alveolar Macrophages on T cell Mitogenesis

Mice were exposed to fly ash of respirable size for 4 weeks (761 $\mu g/m^3$ of air). Alveolar washout cells from fly ash and control animals were removed and added to spleen cell cultures at varying concentrations. Phytohemagglutin was added and cultures were incubated for 24 hours. They were then labelled with ^3H-thymidine for 24 hours and harvested. As seen in the table, normal alveolar cells when added at 10% of the spleen cell concentration caused an enhancement of T cell response to PHA. This enhancement of mitogenesis was significantly less with the addition of fly ash exposed alveolar cells.

	Percent Alveolar Macrophages			
	10%	1%	0.1%	0%
Fly Ash	46,783±2,844*	31,038±749	29,653±819	31,121±400
Control	57,651±2,382	34,530±1,821	32,167±1,564	31,026±638

* Significantly different from control ($P<0.006$).
Data expressed as CPM ± S.E.

These studies were continued and macrophages from fly-ash (1494 $\mu g/m^3$) exposed hamsters were used. The macrophages from the exposed animals were compared to controls. After 70 days of exposure the macrophages from the fly-ash exposed animals had an enhancing effect on T lymphocytes' response to mitogens (PHA) significantly in excess of that found in macrophages from control hamsters. These effects are shown in Figures 26 and 27.

4. Effect of Fly-Ash on Macrophage-Mediated Cytotoxicity

In addition to ADCC we determined the effect of fly-ash on the alveolar macrophage activity against tumor cells, macrophage mediated cytotoxicity (MMC). We found that intratracheal injection of 2 mg of fly-ash into BCG primed hamsters enhanced MMC of pulmonary derived macrophages while a six week course of fly-ash inhalation following BCG activation resulted in a decrease in MMC of the pulmonary-derived macrophages (Figure 28). Fly-ash effects on MMC was in both cases compared to the effects of a second injection of BCG on the MMC of the primed alveolar population.

TABLE 18.

Exposure Time		Number of macrophages with bacteria 0+	1-3	4-6	7 or more
1 week	Fly ash	59.0±3.0	22.7±1.5	9.7±1.9	8.5±1.5
	Control	61.3±2.6	21.2±1.7	8.0±1.3	9.5±1.6
2 week	Fly ash	71.0±1.8	18.7±1.8	7.5±1.0	2.7±0.6
	Control	63.3±2.2*	20.7±1.3	7.6±0.9	9.0±1.9**
3 week	Fly ash	72.6±2.0	18.0±1.5	6.0±1.0	3.5±0.9
	Control	65.2±2.1*	20.8±1.4	7.0±0.9	7.0±1.0*
4 week	Fly ash	77.7±2.3	11.1±1.3	7.7±0.9	3.5±0.9
	Control	64.0±1.8***	17.5±1.0**	10.5±1.0	7.0±1.0*

+ Bacteria per cell, mean ± S.E.

* $p<.05$

** $p<.01$

*** $p<.001$

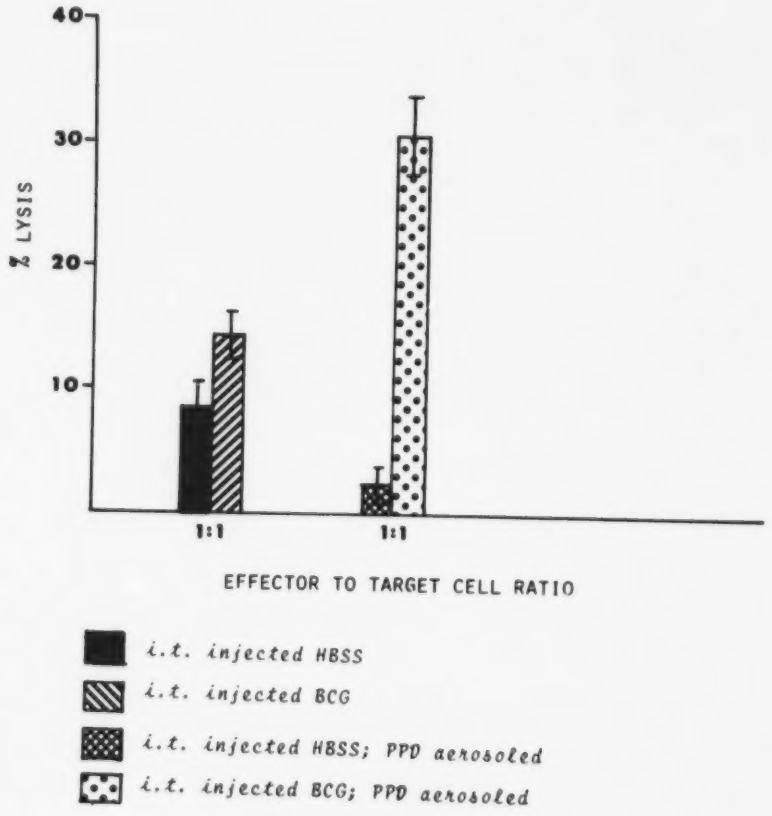


Figure 17. Antibody dependent cell-mediated cytotoxicity of mouse lung cells.

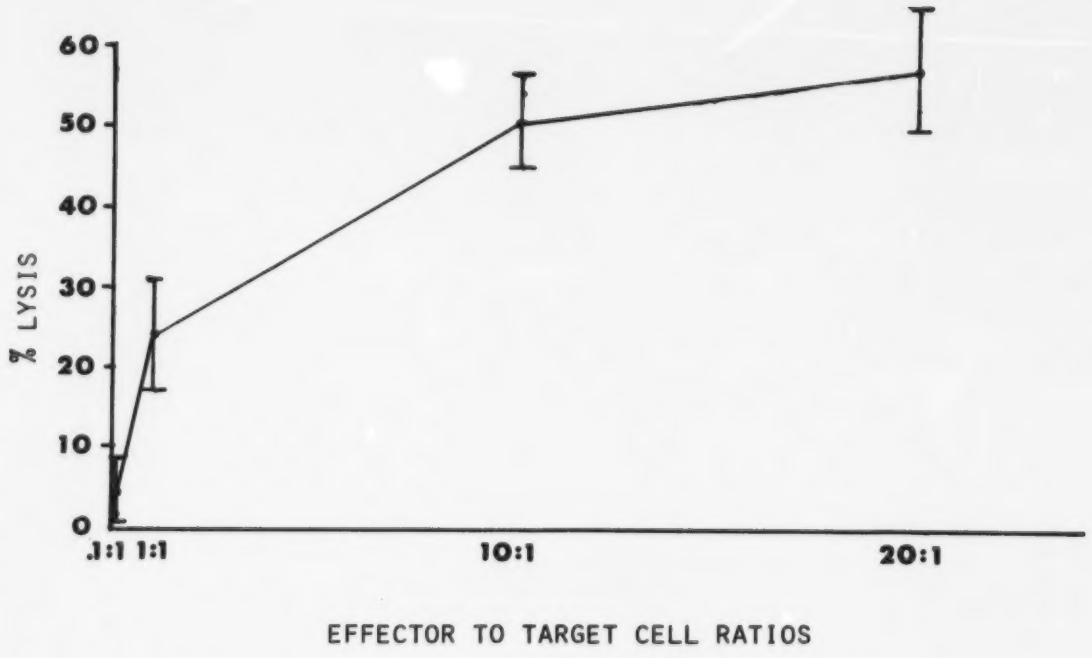


Figure 18. Antibody dependent cell-mediated cytotoxicity of guinea pig lung cells at various effector to target cell ratios.

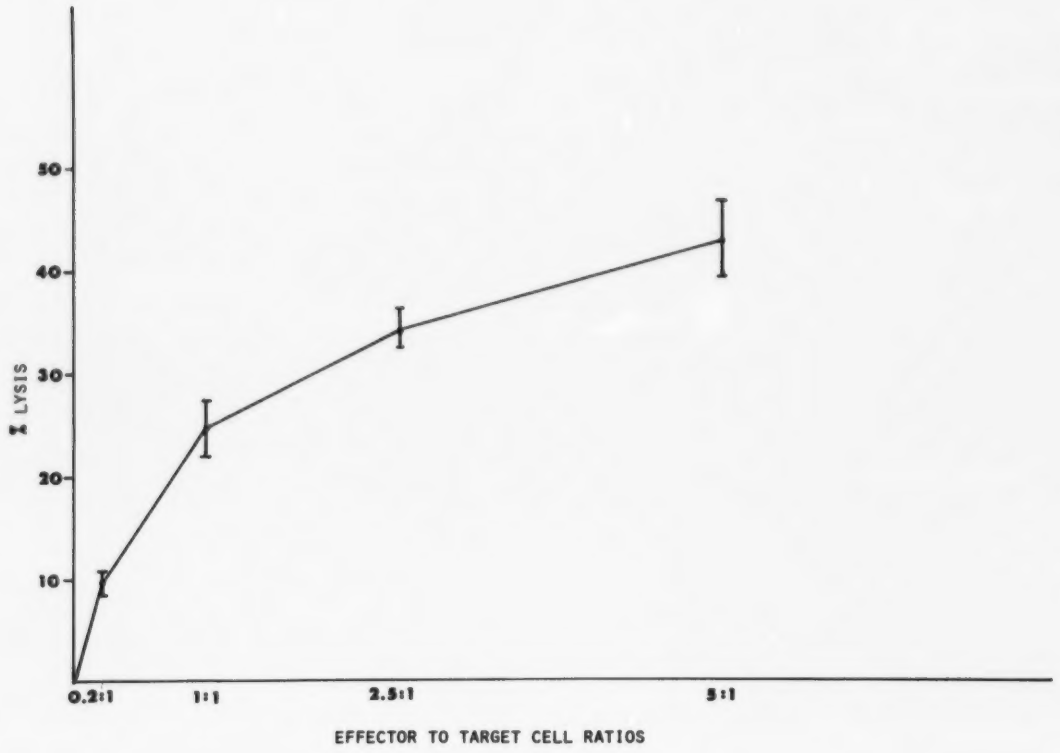


Figure 19. Antibody dependent cell-mediated cytotoxicity of hamster lung cells at various effector to target cell ratios.

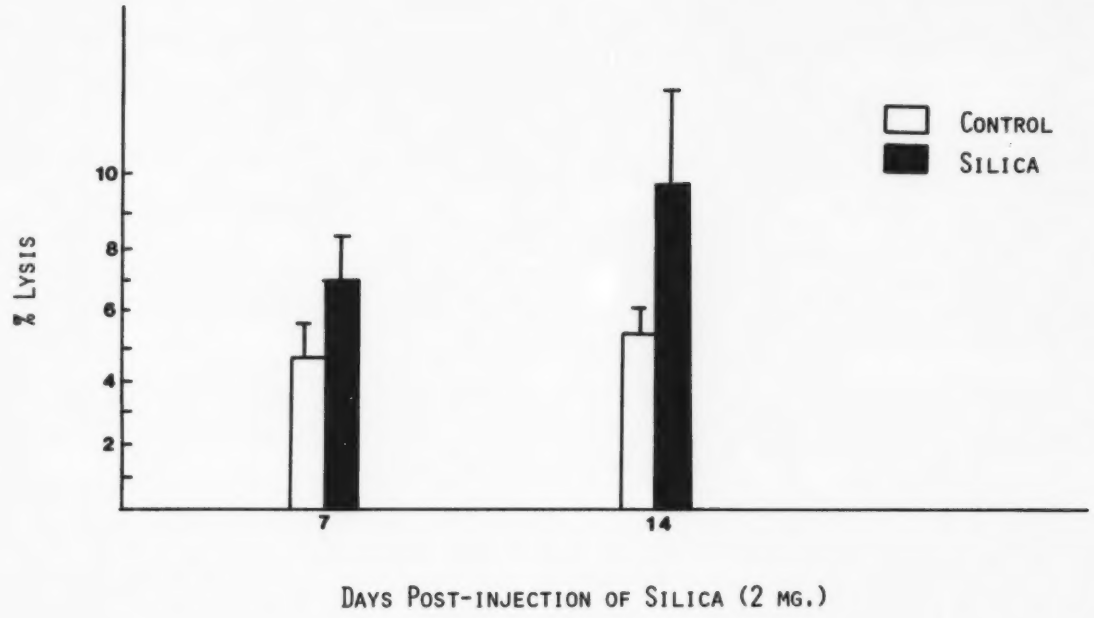


Figure 20. Effects of intratracheal silica injections on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages (1:1 effector/target cell ratio).

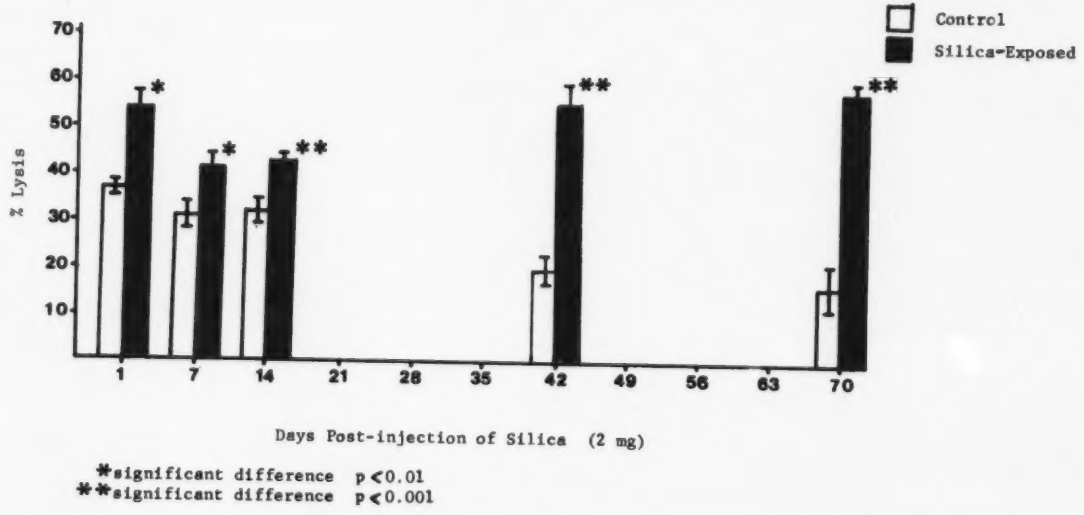


Figure 21. Effects of intratracheal silica injections on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages.

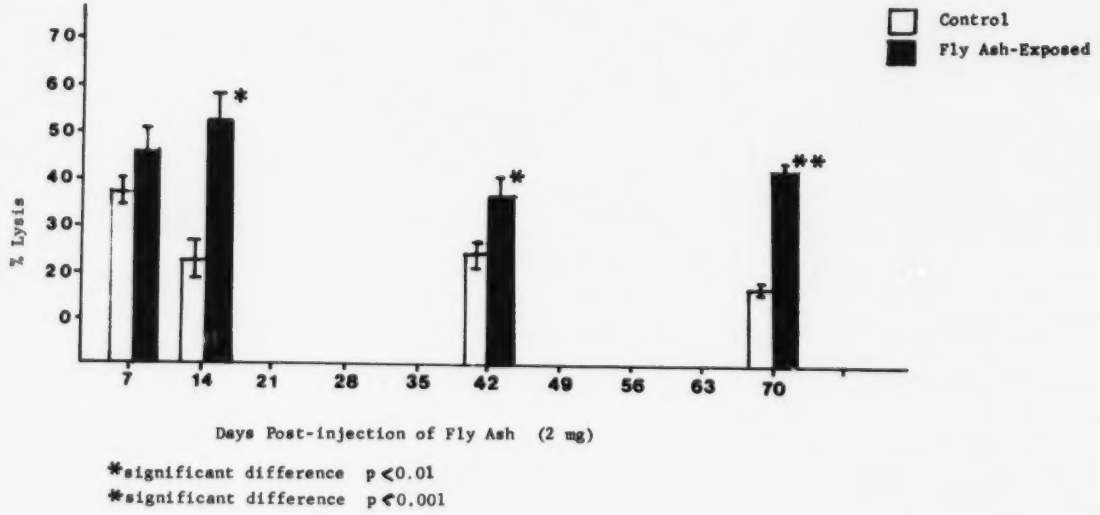
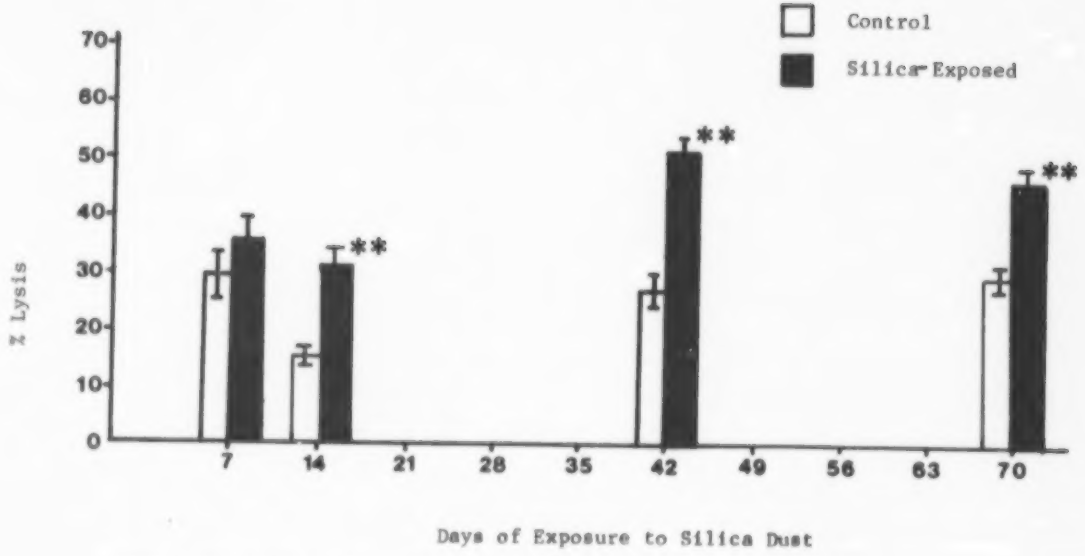
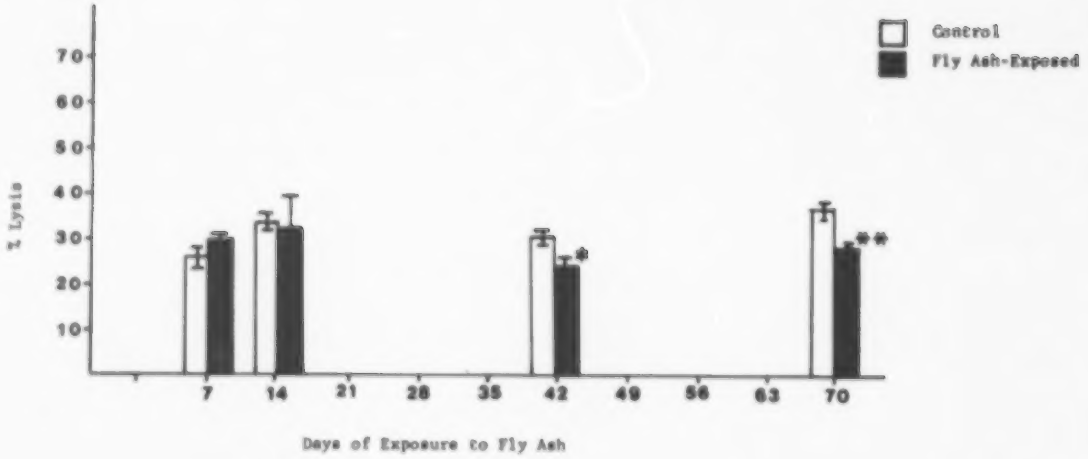


Figure 22. Effects of intratracheal fly ash injections on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages.



**significant difference $p < 0.001$

Figure 23. Effects of silica inhalation on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages.



*significant difference $p \leq 0.01$
**significant difference $p \leq 0.001$

Figure 24. Effects of fly ash inhalation on the antibody dependent cell-mediated cytotoxicity by alveolar macrophages.

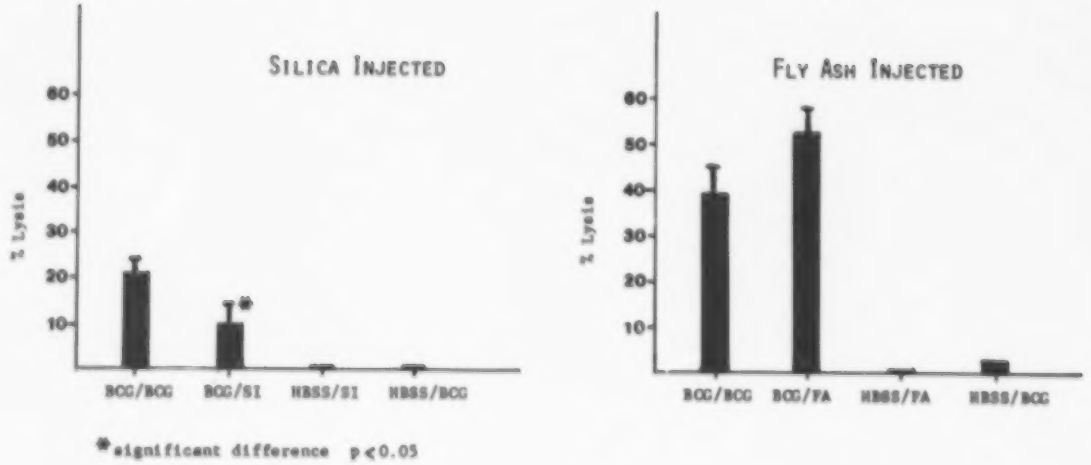


Figure 25. Effects of intratracheal particulate injections on macrophage mediated cytotoxicity.

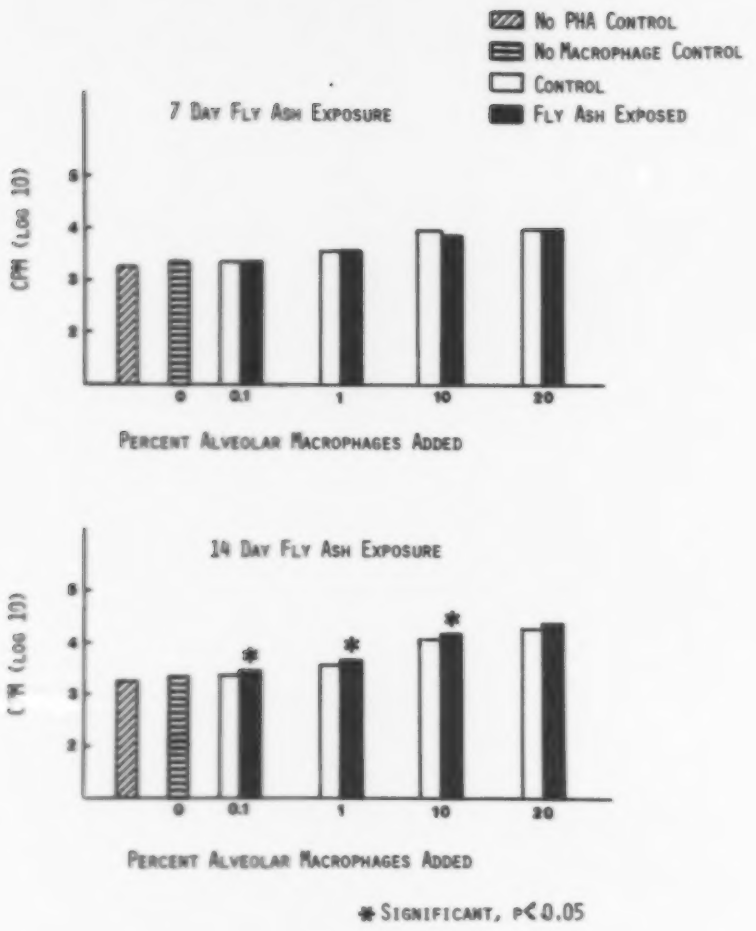


Figure 26. Effects of fly ash inhalation on T lymphocyte mitogenesis with PHA.

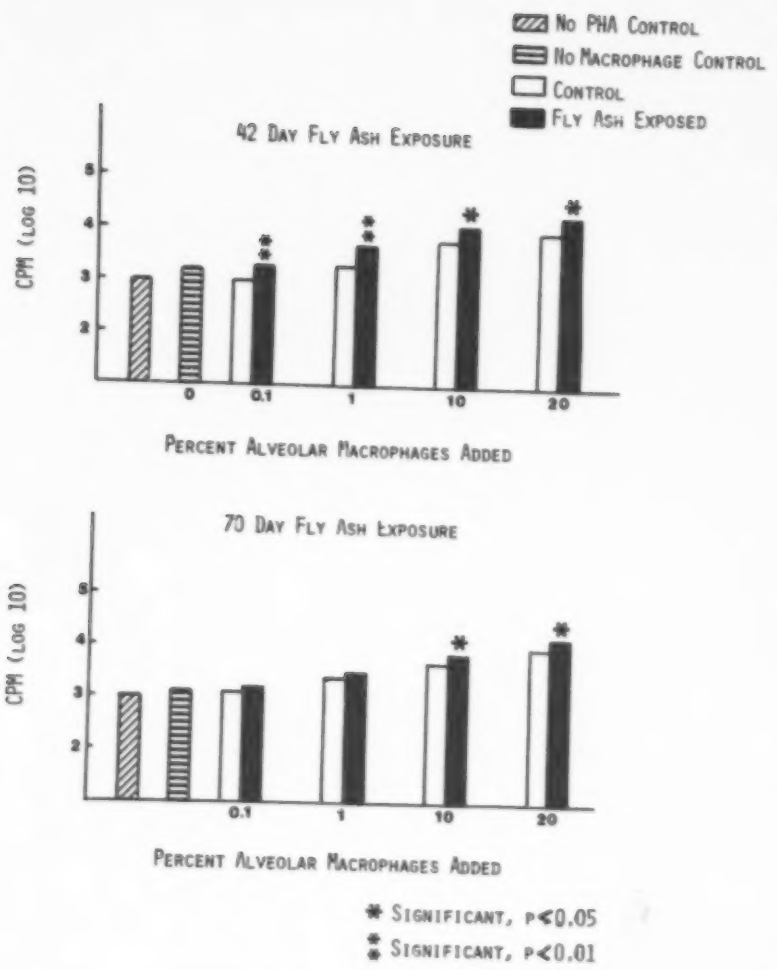
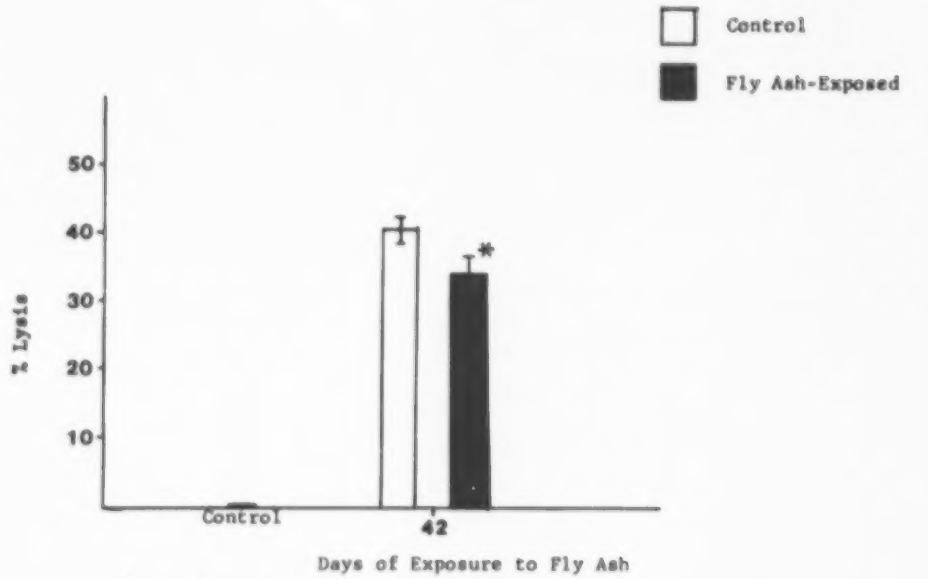


Figure 27. Effects of fly ash inhalation on T lymphocyte mitogenesis with PHA.



*significant difference $p < 0.05$
hamsters received 2 intratracheal injections of BCG 6 wks and 5 days before assay.

Figure 28. Effects of fly ash inhalation on macrophage mediated cytotoxicity by alveolar macrophages.

PART IV

Effect of PSU Fly-Ash Inhalation on Cell-Mediated Immune Response to Sensitizing Antigen

One component of cell mediated immunity is the presence of antigen reactive lymphocytes resulting from initial antigen exposure. These lymphocytes upon reexposure to the sensitizing antigen are stimulated into division and lymphokine production. These responses may parallel the classical delayed type hypersensitivity reactions.

To determine the ability of fly-ash exposed animals to develop this type of cellular immunity the exposed and control mice were injected subcutaneously with BCG in Freund's complete adjuvant. Four weeks after sensitization the blastogenic reactivity to PPD is determined using spleen derived lymphocytes.

In preliminary experiments in our laboratory we found that lymphocytes from BCG sensitized animals were not only more responsive when exposed to PPD but were more active, incorporating more ^{3}H thymidine than lymphocytes from non-BCG stimulated animals in the absence of PPD.

The effects of fly-ash inhalation on this cellular immune response was determined using mice exposed to 1494 $\mu g/m^3$ of fly-ash or respirable size (less than 2.1 μ) for 164 days. The mice, fly-ash exposed and controls, were sensitized with BCG four weeks before removal from exposure chambers. The responsiveness of the lymphocytes to PPD was determined upon removal of the animals from the chambers. The results are shown in Table 19.

TABLE 19.

PPD concentration	Fly ash exposed (N=15)	Control group (N=16)
0	2.82 $\pm$ .04*	2.70 $\pm$ .03
10 μg	3.09 $\pm$ .07	2.94 $\pm$ .04
50 μg	2.99 $\pm$ .05	2.93 $\pm$ .05

* $p < 0.05$.

The results indicate a slightly higher reactivity of lymphocytes obtained from fly-ash exposed animals than that found in controls. Further studies would be required to determine whether these observations are consistent with fly-ash exposure and whether longer exposures would have a potentiating effect on cellular immune sensitization.

Effect of Fly-Ash Inhalation on Delayed-Type Hypersensitivity Response in the Lung

Another measure of cellular immunity is the delayed type hypersensitive response. This response is characterized by the infiltration of mononuclear cells to the area of antigen deposition in a sensitized animal. Release of lymphokines also leads to an inflammatory response and an accumulation of fluid as well as cells. Under experimental conditions the challenge application is normally applied intradermally and the degree of swelling can be measured. Since the lung is not readily accessible for manipulation, we used a method adapted from a footpad technique described by Schwartz et al. (J. Immun. 118 (1977):159-165. In this method mice are sensitized by subcutaneous injection of BCG in complete adjuvant. Twenty-one to 28 days later the mice are injected intravenously with radiolabelled mononuclear cells obtained from unsensitized syngeneic mice. A few hours later the sensitized mice are challenged with an intratracheal injection of PPD. 48 hours later the lungs are removed and the accumulation of radioactive cells in the lungs is determined by measuring the amount of radioactivity present in the lungs as a ratio of the total radioactivity injected. The specificity of this method is determined by using several controls as shown in Table 20.

TABLE 20.

Group (N)	BCG sensitization	PPD challenge	% of total radioactivity Incorporated ± S.E.M.
1(5)	+	+	3.02 ± .30*
2(5)	+	-	1.50 ± .11
3(5)	-	+	1.31 ± .07
4(5)	-	-	1.12 ± .04

* $p < 0.001$ compared to other groups.

The results of the preliminary experiments indicated that there is a relationship between the degree of radiolabelled cell infiltration and sensitivity to an antigen. Several antigens were used in the studies. Generally, incorporation of radioactivity in excess of 2% is indicative of sensitivity of the pulmonary tissues.

The effect of 160 day fly-ash inhalation on pulmonary sensitization were determined. The average concentration of particles less than 2.1 μ was 1494 $\mu g/m^3$ during this period. The mice were sensitized with BCG four weeks prior to this removal from exposure chambers. They were injected intratracheally with PPD upon removal from the chambers. The results are shown in Table 21.

TABLE 21.

Group (N)	Percent of ^{51}Cr incorporation in lungs $\pm$ S.E.M.
Fly ash (23)	3.12 $\pm$ .15
Control (24)	2.67 $\pm$ .21

These results indicate that the mice exposed to fly-ash become more sensitive to BCG. Though the increase in cellular reaction was not statistically significant it is consistent with the in vitro response of spleen cells to PPD that was obtained in the parallel group of fly ash exposed animals.

Based on the results obtained in these studies we feel that fly ash exposure does enhance the animals' ability to be sensitized to certain antigens and respond more vigorously with a cellular reaction than animals housed in control exposure chambers. Whether these cellular reaction would be protective or lead to tissue damage we are not in a position to speculate based on such preliminary information.

PART V

Examination of Alveolar Macrophages by Electron Microscopy and Energy Dispersion X-Ray Analysis (EDXA)

Mice were maintained in exposure chambers for 1, 2, 3 and 4 weeks and exposed to PSU fly ash at 2577±318 ug/m^3 of air with 25.72±3.24% of the particles being < 2.1 um in diameter and 68.36±5.56% being < 4.7 um. Control mice were maintained in identical chambers but inhaled filtered air. Groups of two, ten and eight mice were removed from the chambers at each time period for scanning electron microscopy, transmission electron microscopy and a phagocytic study respectively.

Differences between the viability of fly ash-exposed macrophages and control macrophages as determined with trypan blue absorption were not statistically significant. Results are shown in Table 22.

As shown in Table 23, in-vitro alveolar macrophage cultures from mice exhibited a significantly depressed activity to phagocytize Staphylococcus aureus after the inhalation of fly ash for 2, 3 and 4 weeks, compared to controls.

Fly ash samples are to be analyzed by the Environmental Protection Agency (analysis in progress) utilizing spark source mass spectrometry (SSMS) with isotope spiking and direct optical emission spectroscopy (OES) to determine the concentration of elements. Energy-dispersive X-ray analysis (EDXA) was used to determine which elements were present in respirable-sized fly ash particles utilized during this study. All elements found when using EDXA were also found when using SSMS and OES on a previously used sample, but the reverse was not true. However, all of the major elements composing fly ash were found when utilizing EDXA.

Energy-Dispersive X-Ray Analysis (EDXA)

Scanning Electron Microscopy (SEM)--Alveolar macrophages from mice exposed and not exposed to fly ash were analyzed utilizing EDXA in the SEM mode. These results are shown in Table 24. Elements are listed in decreasing concentrations with a few exceptions. Sodium was present in fly ash at 0.5-1.0% of the total amount. Therefore, it was assumed to be present, but it was not certain due to the presence of other elements in the same energy range. In weeks one and three arsenic and lead were present, however, their relative concentrations could be determined because both of their K_α peaks were within the same energy range.

Transmission Electron Microscopy (TEM)--Alveolar macrophages from mice exposed and not exposed to fly ash were also analyzed using EDXA in the TEM mode. These results are shown in Table 25 with elements listed in decreasing concentrations. The same problem arose when analyzing for sodium (see EDXA:SEM).

Transmission and Scanning Electron Microscopy

Initially, control cells were observed in both the transmission and scanning modes to establish the normal ultrastructure and surface morphology of murine alveolar macrophages. The ultrastructure of control alveolar macrophages displayed such a large amount of variation that characteristic alveolar macrophages could not be ascertained. Variations were observed in the amount of mitochondria, endoplasmic reticulum, phagosomes, lysosomes, secondary lysosomes and myelin figures and in the shape of nuclei.

Scanning electron microscopy also revealed large amounts of variation between control cells. These variations included differences in cell shape, size, membrane ruffling and cytoplasmic projections.

All of the ultrastructural and morphological differences observed when analyzing control cells were also seen when analyzing cells exposed to fly ash at all exposure periods.

TABLE 22.

Exposure Time	% viable macrophages Fly ash	Control
1 week	87.24±3.42*	90.24±4.35
2 weeks	80.59±2.45	77.45±2.89
3 weeks	82.24±2.32	84.92±2.47
4 weeks	86.74±4.29	84.22±3.67

* Mean ± S.E.

N = 6.

TABLE 23.

Exposure Time		% of macrophages Containing S. aureus
1 week	Fly ash	41.00±3.05*
	Control	38.63±2.69
2 weeks	Fly ash	29.00±1.89**
	Control	36.67±2.20
3 weeks	Fly ash	27.33±2.04**
	Control	34.71±2.16
4 weeks	Fly ash	22.29±2.35**
	Control	36.00±1.86

* Mean ± S.E.

** $p < 0.05$.

N = 8.

TABLE 24.

Fly ash	Control	F.A.-Cont.	Cont.-F.A.
Week 1			
Si	Si	Si	
Al	Ca	Al	
Fe	Fe	Fe	
Ca	Al	Ca	
K	Ti	K	
Zn	Zn	Zn	
Ti	K	Ti	
S	S	S	
Mg	Mn	Mg	
Cu	V	Cu	
Mn	Cr	Mn	
Ni	Co	Ni	
V	Cu	V	
Cr	Mg	Cr	
Co		Co	
	Na*		
Na*		Na*	
As*		As*	
Pb*		Pb*	
Week 2			
Si	Si	Si	
Al	Mg	Al	
Fe	K	Fe	
P	Al	P	
Ca	Fe	Ca	
K	Cu	K	
Mg	Zn	Mg	
Zn	Mn	Zn	
Cu	Ti	Cu	
Ti	Co	Ti	
Mn	V	Mn	
V	Cr	V	
Co		Co	
Cr	Na*	Cr	
Na*		Na*	

(continued)

TABLE 24 (continued)

Fly ash	Control	F.A.-Cont.	Cont.-F.A.
Week 3			
Si	Si	Si	
Al	K	Al	
Fe	Al	Fe	
Ca	Ca	Ca	
K	Fe	K	
Ti	Zn	Ti	
Mg	Ti	Mg	
Zn	Mg	Zn	
Cu	V	Cu	
Mn	S	Mn	
Cr	Cr	Cr	
V	Cu	V	
S	Co	S	
Co	Mn	Co	
Na*	Na*	Na*	
As*		As*	
Pb*		Pb*	
Week 4			
Si	Si	Si	
Al	Al	Al	
Fe	Ti	Fe	
K	Zn	K	
Ca	Fe	Ca	
Ti	K	Ti	
Mg	Mg	Mg	
Zn	Ca	Zn	
Cu	Mn	Cu	
As	Co	As	
Cr	S	Cr	
Mn	Cr	Mn	
Co		Co	
S	Na*	S	
Na*		Na*	

TABLE 25.

Fly ash	Control	F.A.-cont.	Cont.-F.A.
Week 1			
Si	Si	Si	
Al	Cu	Al	
Cu	Al	Cu	
Fe	S	Fe	
K	Fe	K	
Ca	Mg	Ca	
S	Ca	S	
Mg	K	Mg	
Na*	Na*	Na*	
Week 2			
Si	Si	Si	
Al	Cu	Al	
Cu	Mg	Cu	
Fe	Fe	Fe	
K	Al	K	
P	Ca	P	
Ca	K	Ca	
Ti		Ti	
Mg	Na*	Mg	
Na*		Na*	
Week 3			
Si	Si	Si	
Cu	Cu	Cu	
Al	Al	Al	
Fe	S	Fe	
Ti	Fe	Ti	
K	Mg	K	
Ca	Ca	Ca	
Mg	K	Mg	
S		S	
	Na*		
Na*		Na*	

(continued)

TABLE 25 (continued)

Fly ash	Control	F.A.-cont.	Cont.-F.A.
Week 4			
Si	Si	Si	
Al	Cu	Al	
Cu	Al	Cu	
Fe	Fe	Fe	
Ca	S	Ca	
K	Mg	K	
Ti	Ca	Ti	
Mg	K	Mg	
S	Ti	S	
Na*	Na*	Na*	

* Other elements are also present in the Na range making comparisons difficult.

PART VI - Histological Examination of Tissues From Fly-Ash Exposed Mice

Wet tissues from 48 female mice were processed, embedded in parafin and sectioned at 6 microns. Slides were stained routinely with Hematoxylin and Eosin and occassionally with Prussian Blue for the presence of iron. The following tissues were observed by ordinary light and polarizing microscopy:

1) lung
2) trachea
3) mediastinal lymph node
4) spleen
5) liver
6) Peyer's patches of the small intestine
7) mesenteric lymph node

All lesions were graded and characterized according to the following scheme:

\+ = minimal
++ = mild
+++ = moderate
++++ = severe
Abs = tissue absent
\- = no lesion present in tissue (normal)

RESULTS

SHORT TERM EXPOSURE - SIX WEEKS

Trachea

No lesions were present in the trachea.

Lung

Pigmented alveolar macrophages (PAMA) were present in all lungs of Group I animals. These macrophages were randomly distributed in the lung parenchyma and could also be found along the mucosal surface of the bronchi. When viewed under ordinary light, they contained a black granular pigment. Under polarized light, fine birefringent crystals could also be observed within the cytoplasm of the pigmented macrophage. The presence of iron could not be demonstrated by the Prussian Blue reaction. Eight of 12 lungs examined contained multiple foci of lymphoid accumulation near the terminal

bronchioles. One animal in Group I and II had a single inflammatory lesion. This lesion was diagnosed as focal peribronchiolitis. This was considered an incidential finding.

Mediastinal Lymph Node

Pigment deposition was present in minimal amounts in the sinusoids of 5 out of 10 mediastinal lymph nodes examined.

Ileum (Peyer's Patches)

No lesions were present in the ileum (Peyer's patches).

Mesenteric Lymph Node

No lesions were present in the mesenteric lymph node.

Spleen

No lesions were present in the spleen.

Liver

No lesions were present in the liver.

LONG TERM EXPOSURE - 31 WEEKS

Trachea

A mild chronic tracheitis was observed in one animal in Group III (#36).

Lung

In Group III all lungs examined contained pigmented alveolar macrophages. As in the short term exposure, a few birefringent particles could be demonstrated under polarized light. These pigmented alveolar macrophages were found along the bronchial mucosa and observed in clusters in peribronchiolar areas. Only 3 of 12 animals had PAMA in significant numbers in peripheral alveolar areas. Lymphoid accumulation near the terminal bronchioles was more prominent in treated vs matched control animals and also more prominent than that observed in Groups I and II. Focal peribronchiolitis was observed in one animal from Group III and one from Group IV. The latter findings were considered incidental.

Mediastinal Lymph Node

In Group III, pigment deposition was more prominent (++) than that observed in Group I animals.

Ileum (Peyer's Patches

No lesions were observed in the ileum (peyer's patches).

Mesenteric Lymph Node

No lesions were observed in the mesenteric lymph node.

Spleen

No lesions were observed in the spleen.

Liver

No lesions were observed in the liver.

SUMMARY AND CONCLUSIONS

1. Pigmented alveolar macrophages were observed in the lungs of mice in both short and long term exposures. The black non-birefringent pigment resembled anthracotic pigment. Particles which were birefringent resembled silica.

2. In the short term exposure lungs, pigment was more randomly dispersed than those subjected to long term exposure. In the long term exposure lungs, pigment was more prominent and tended to localize in alveoli immediately adjacent to terminal bronchioles.

3. Uptake of pigment by mediastinal lymph nodes was more intense in animals from long term exposure.

4. Other reticuloendothelial tissues, i.e., Peyer's patches, mesenteric lymph nodes, spleen and liver (Von Kupffer's cells) did not show evidence of fly ash pigment uptake.

5. Foci of lymphoid accumulation which was associated with fly ash pigment deposition was observed to be in greater amount in the long term exposure. In both exposure times, this finding is interpreted as a reaction to the presence of fly ash pigment.

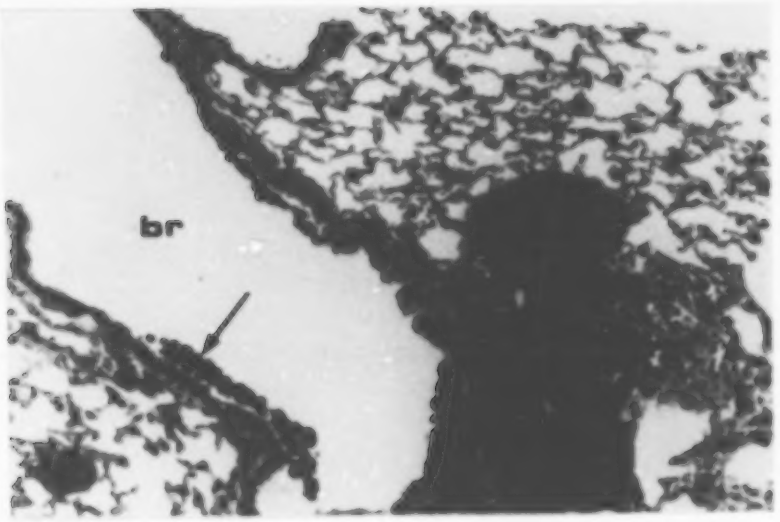


Figure A. Section of lung through bronchiole(br) showing accumulation of PAMA (small arrow) along epithelium. Also shown is an accumulation of lymphoid cells (parabronchial lymphoid infiltration) to bronchiolar epithelium (large arrow). x100, #80-35.

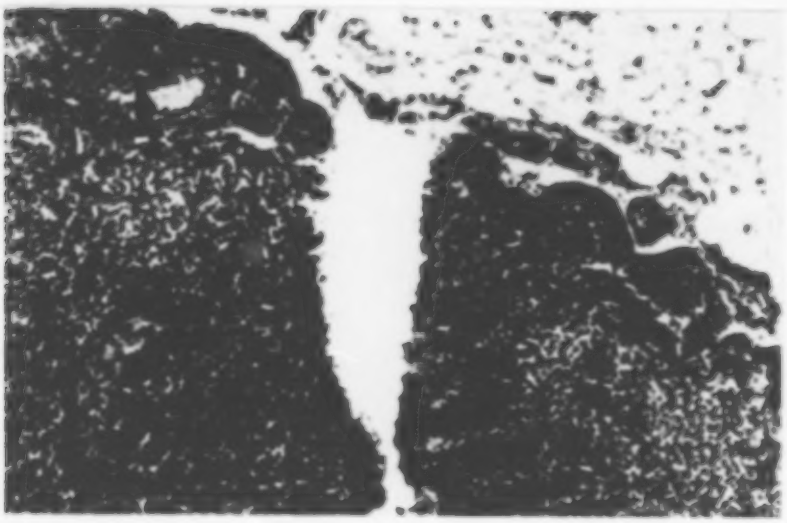


Figure B. Section through mediastinal lymph node. Note accumulation of dark pigment in peripheral portion of lymph node (arrow). x100, #80-35.

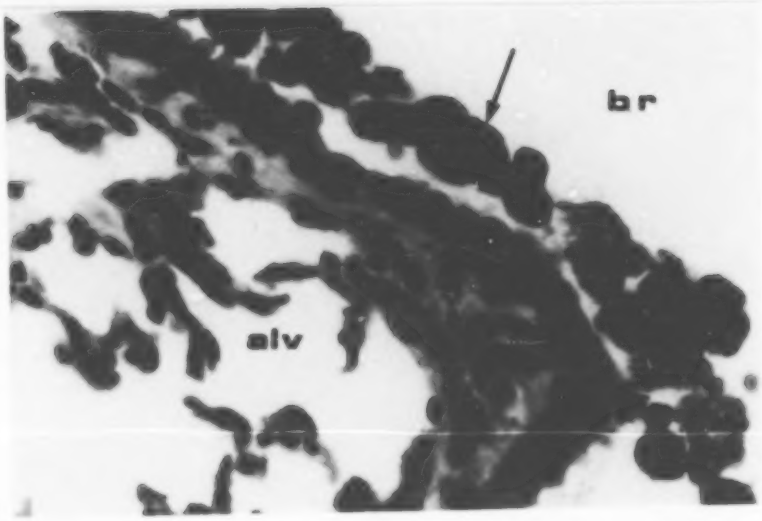


Figure C. A higher magnification of figure A, showing the relationship of PAMA (arrow), the lining epithelium, and a normal alveolus (alv). x400.

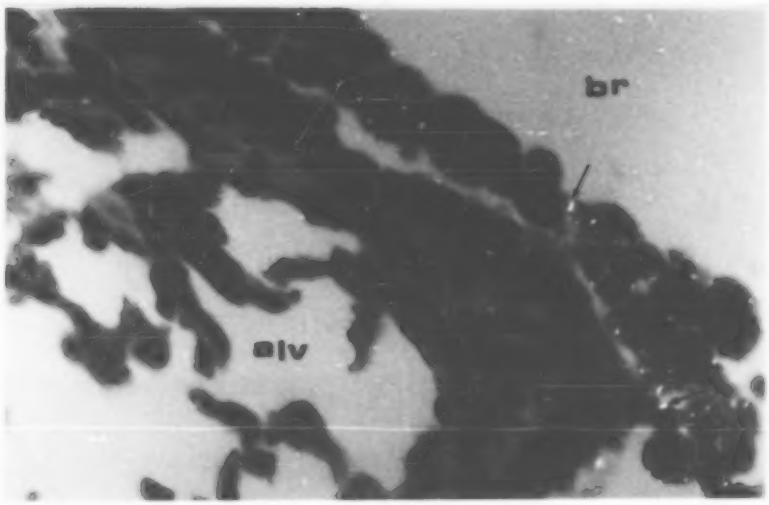


Figure D. Same section as figure C, above, but examined under polarized light. Note birefringent particles in periphery of PAMA (arrows). x400.

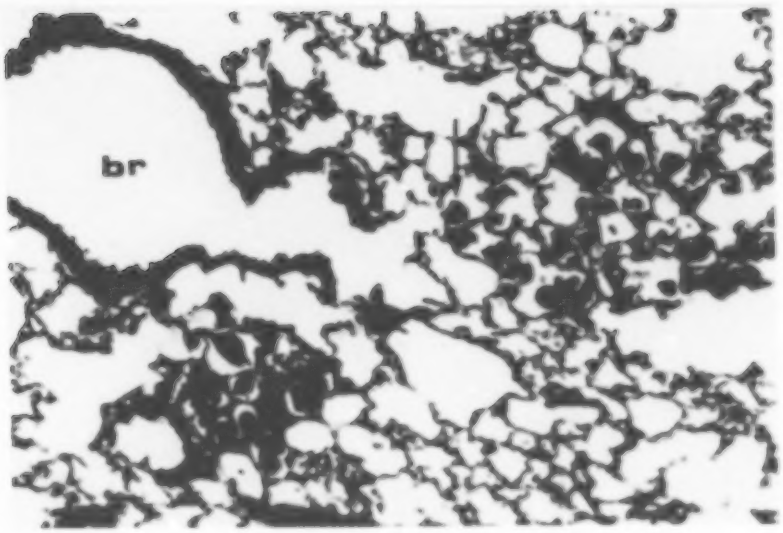


Figure E. Section of lung showing deposition of PAMA (arrow) in Alveoli near terminal bronchiole(br). x100.

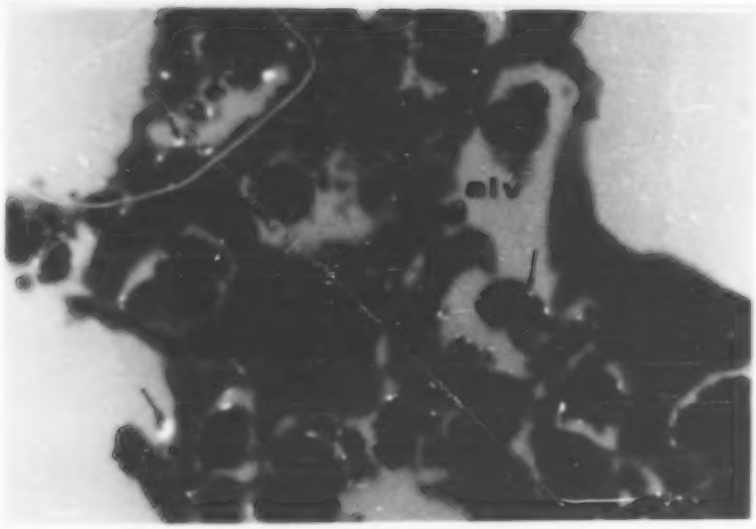


Figure F. A higher magnification of figure E, above, observed under polarized light. Note presence of birefringent particles in PAMA (arrows). x400.

SUMMARY

Exposure of mice to relatively high concentrations of fly-ash resulted in alterations in immunological functions of lymphocytes and macrophages and also progressive lesions in lungs and mediastinal lymph nodes. Fly-ash inhalation changed the ability of mice to respond to subsequent E. coli antigen introduced by inhalation. This change, a decreased response in the spleens and mediastinal lymph nodes was not large until after nine months of exposure. This was in contrast to a very rapid and severe suppression occurring after carbon black and silica quartz inhalations.

Fly-ash had little, if any, effect on the ability to T and B lymphocytes to respond to mitogens (PHA and LPS) and to be stimulated for cytolytic response against tumor cells. This lack of change in T cell functions also was found after exposures to carbon black.

The effects of fly-ash on macrophages was more pronounced: a decrease in the number of pulmonary macrophages capable of phagocytosis; a decrease in antibody dependent cytolysis (ADCC) after 42 and 70 days of exposure, as contrasted to enhancement in ADCC after intratracheal injection of fly-ash and intratracheal injection and inhalation of silica; an increase in cytotoxic activity against H50 tumor cells after IT injection of fly ash and a decrease after fly ash inhalation; a decrease ability to activate T cell mitogenesis after fly ash inhalation.

Fly-ash exposures were enhancing to cellular immune responses. Both the in-vitro response of the sensitized mice to PPD and the in-vivo delayed-type hypersensitive reactions were increased.

Histological examination of the tissues from mice exposed for 42 and 217 days indicated that fly ash was localized primarily in macrophages present in lungs and mediastinal nodes. The lesions, macrophage and lymphoid cell accumulations, were peripheral in the lung after the shorter exposure and more central after the longer exposure. The lesions were less severe than those obtained after equivalent exposures to silica quartz.

CONCLUSIONS

Fly-ash in relatively high concentrations has little effect on anti-microbial and antitumor activities of lymphocytes and macrophages. Inflammatory changes suggestive of early pneumoconiosis were seen in the lungs after 217 days of exposure to fly ash.

APPENDIX

The fly-ash used at Penn State in Series I and II Experiments was analyzed by EPA. The following tabulation presents the results:

Al	12 %	Mo	100 ppm
As	700 ppm	Nb	20 ppm
B	300 ppm	Ni	350 ppm
Ba	750 ppm	P	3000 ppm
Bi	20 ppm	Pb	300 ppm
Ca	2 %	Ru	300 ppm
Cd	1 ppm	Sb	30 ppm
Co	50 ppm	Si major constituent	> 20 %
Cr	280 ppm	Sn	< 10 ppm
Cs	80 ppm	Sr	900 ppm
Cu	350 ppm	Th	3 ppm
Fe	8.0 %	Ti	6400 ppm
Ga	50 ppm	Ta	50 ppm
In	2 ppm	U	12 ppm
La	60 ppm	V	200 ppm
Mg	∿ 2500 ppm	Zn	800 ppm
Mn	300 ppm	Zr	50 ppm

The E.P.A. supplied fly-ash was characterized by Oak Ridge National Laboratory. The median geometric diameter of the particles was 1.8 μm. Major elements present were As, Cu, Nb, Pb, Rb, Sr, Ti, V, Y, Zn and Zr. Seven polycyclic organic compounds (BP, chrysene, benz(a)anthracene, pyrene, phenanthrene, fluoranthene and anthracene) were also identified.